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STUDIES ON CALCIUM PHOSPHATE FATTY ACID SALTS OCCURRING IN FECAL LIPIDS[1]

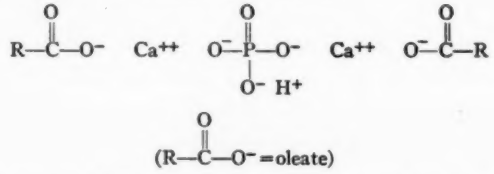
J. F. RICHARDS[2] AND K. K. CARROLL

Abstract

The presence of unsaturated fatty acids such as oleic, linoleic, eicosenoic, and erucic acid in the diet of rats causes a marked increase in lipid-soluble calcium and phosphorus in the feces. A proposed structure of a calcium phosphate fatty acid complex salt for this material has been confirmed by chemical synthesis and characterization by paper chromatography.

Introduction

It was reported by Kim and Ivy (1) and Pihl (2) that rats fed a diet containing oleic acid excreted large quantities of phospholipid in their feces. Further studies by Swell, Trout, Field, and Treadwell (3, 4) showed that this was not a true phospholipid but rather a calcium oleate phosphate complex for which they proposed the following structure:



The material was purified by precipitation from chloroform by acetone or methanol and, after several repetitions of this procedure, a product with chemical composition approaching the theoretical was obtained. However, a product with constant composition could not be isolated and if the precipitations were continued, the calcium and phosphorus content of the lipid increased above the theoretical and the fatty acid content decreased to values lower than the theoretical. The substance was readily decomposed by acid and dissociation curves indicated a pK of 6.0 to 6.3. The infrared spectrum was in accordance with the proposed structure.

Studies in our laboratory have shown that ingestion of other unsaturated fatty acids such as linoleic and erucic acids also produce large increases in the

[1]Manuscript received January 10, 1959.
Contribution from the Collip Medical Research Laboratory, University of Western Ontario, London, Ontario.
[2]Holder of studentships from the National Research Council of Canada (1956–58).

lipid-soluble phosphorus and calcium excreted by rats (5). This suggested the formation of complex salts similar to the one described by Swell *et al.* for oleic acid. Further evidence for the existence of such substances has been obtained by the use of paper chromatography and by developing a chemical method for their synthesis. This work forms the basis of the present report.

Methods and Results

Effect of Dietary Lipid on Fecal Excretion of Complexes

Previously published results from this laboratory showed that dietary oleic, linoleic, and erucic acids all caused a marked increase in the amount of lipid-soluble phosphorus and calcium in the feces of rats (5). Trierucin gave a response similar to that of free erucic acid but triolein and trilinolein had no effect. Further experiments have been carried out using the experimental conditions described previously, and these have shown that eicosenoic acid is as active as oleic and erucic acids in promoting the excretion of lipid-soluble phosphorus and calcium but nervonic acid has very little effect (Table I).

TABLE I

Fecal excretion of lipid-soluble phosphorus and calcium

Dietary fatty acid	Lipid-soluble phosphorus (mg)	Lipid-soluble calcium (mg)
Oleic	183	768
Eicosenoic	239	422
Erucic	116	363
Nervonic	11	40
Linoleic	79	283
Palmitic	3	0
Stearic	6	0

NOTE: The fatty acids were fed at the 10% level in synthetic diets to groups of three rats. The feces were collected for two 7-day periods and aliquots were extracted with ether in a Soxhlet apparatus as described previously (5). The figures represent total excretions for the group over a 14-day period.

Saturated fatty acids and a preparation of cerebrosides from beef brain were also ineffective. The eicosenoic acid for these experiments was prepared by fractional distillation of rapeseed oil fatty acids and was generously provided by Dr. B. M. Craig, Prairie Regional Laboratory, Saskatoon. The nervonic acid was prepared synthetically (6). The cerebroside preparation was provided by the Wilson Laboratories, 4221 South Western Avenue Blvd., Chicago 9, Illinois.

Paper Chromatography

Lipid extracts from feces of rats fed synthetic diets containing 10% oleic or erucic acid (5) were applied to Whatman No. 1 filter paper strips which had been dried at 100° C for 1 hour. The chromatograms were developed, ascending, for 2 hours with petroleum ether (boiling range 38–50° C) – methanol – chloroform in the proportions 80:15:5 or 85:10:5 by volume. The solvents were reagent grade, and were used without further purification except that the chloroform was washed with 10% aqueous sodium bicarbonate. The fecal

extracts were spotted in triplicate and one strip was used for the detection of phosphorus, one for calcium, and one for fatty acid. Phosphorus was detected by the method of Hanes and Isherwood (7), calcium by spraying with 1% hematoxylin in 90% ethanol, and unsaturated fatty acid by exposing the strip to iodine vapor (8). Results are shown in Table II. The presence of complex

TABLE II

Paper chromatography of fecal lipid extracts

Material applied	R_f values		
	Calcium	Phosphorus	Fatty acid
A. Petroleum ether – methanol – chloroform (80:15:5)			
Oleic extract	0.52, 0.85	0.51	0.49, 0.84
Erucic extract	0.43, 0.78	0.41	0.41, 0.79
Calcium oleate	1.0		1.0
Calcium erucate	1.0		1.0
B. Petroleum ether – methanol – chloroform (85:10:5)			
Oleic extract	0.31, 0.96	0.96	0.31, 0.97
Erucic extract	0.12, 0.22	0.21	0.15, 0.25
Calcium oleate	0.32		0.32
Calcium erucate	0.11		0.12
C. Petroleum ether – methanol – methylene chloride (85:10:5)			
Oleic extract	0.19, 0.91	0.92	0.17, 0.91
Erucic extract	0.19, 0.91	0.90	0.18, 0.90
Calcium oleate	0.20		0.19
Calcium erucate	0.21		0.19

NOTE: Free oleic and erucic acid ran at the solvent front in each of these systems. Calcium phosphate gave a streak at the origin.

in the fecal extracts is indicated by the occurrence of calcium, phosphorus, and fatty acid spots with the same R_f values. Extra calcium and fatty acid spots apparently indicate the presence of calcium soaps although the agreement with controls in system A is not very good.

The above chromatographic systems have some disadvantages. The solvent front did not move very far in 2 hours; usually 6–10 cm in the 80:15:5 solvent mixture. If allowed to run more than 3 hours, the complex seemed to dissociate, as shown by trailing of the calcium and phosphorus spots, rather than the usual close overlapping. If the chloroform in the system was replaced by methylene chloride or ethylene dichloride, the chromatograms could be developed for 4–5 hours without dissociation of the complex and the solvent front advanced 15–20 cm. Some results with the methylene chloride system are given in Table I and similar R_f values were obtained with ethylene dichloride.

In one study, ether extracts of feces of rats fed 10% oleic or erucic acid diets were applied to chromatograms and developed for 5 hours in a petroleum ether – methanol – methylene chloride (80:15:5) system. The strips were subjected to iodine vapor and the spots corresponding to the complex were cut out and eluted with petroleum ether. The eluates were transferred to digestion tubes and, after the solvent had been removed, the residue was oxidized with 60% perchloric acid. Determinations of calcium (9) and

phosphorus (10) were performed on the digested material. The calcium: phosphorus ratio was found to be 1.96:1 in the case of the oleic acid fed rats and 2.13:1 for the erucic acid fed rats. These are in good agreement with the ratio of 2:1 required for the proposed formula of the complex salts.

It seems probable that the fatty acid component of the complex is in each case derived from the dietary fatty acid. The complex derived from the feces of erucic acid fed rats was purified by several precipitations from chloroform–methanol and decomposed with acid. When the fatty acid fraction was separated and chromatographed on paper (8), it showed only one spot with an R_f identical with that of erucic acid.

Chemical Synthesis

The sodium salts of oleic, erucic, and palmitic acids were prepared by neutralizing the free acids with sodium hydroxide in dilute aqueous solution. The calcium salts were then prepared by addition of an aqueous calcium chloride solution. They were collected by filtration, washed free of chloride, and dried *in vacuo* to constant weight at room temperature (11). The calcium salts were dissolved in *n*-butanol (10% w/v) with warming and the theoretical amount of phosphoric acid in *n*-butanol (0.5% v/v) was added dropwise to the hot stirred solution. A precipitate appeared in each case and was collected by centrifugation. This was suspended in chloroform and the mixture was again centrifuged to remove insoluble calcium phosphates. The clear chloroform supernatant was transferred to another centrifuge tube and the complex salt was precipitated by the addition of four volumes of methanol. This precipitation was repeated several times and the precipitates were then dried and analyzed for calcium and phosphorus. It can be seen from the results in Table III that after five precipitations the complex salts formed with oleic acid and palmitic acid gave analytical figures very close to the theoretical but three precipitations of the salt formed with erucic acid were apparently insufficient. The yield of complex salts was 20–40% of the theoretical.

TABLE III

Analysis of complex salts prepared synthetically

Material	% Phosphorus		% Calcium	
	Found	Calculated	Found	Calculated
Calcium oleate phosphate	4.3	4.2	11.0	10.8
Calcium palmitate phosphate	4.1	4.5	11.5	11.7
Calcium erucate phosphate	5.1	3.7	12.2	9.4

NOTE: The oleate and palmitate complexes were precipitated five times, the erucate complex only three times.

The synthetic complexes containing oleic acid or erucic acid had solubility properties very similar to those of the substances found in fecal extracts, and their behavior in the paper chromatographic systems described above was identical with that of their biologically formed counterparts. The synthetic complex prepared from palmitic acid was much less soluble than those prepared from unsaturated acids and remained at the origin in the paper chromatograms.

Discussion

The results presented provide further evidence for the structure proposed by Swell *et al.* (4) for the calcium and phosphorus-containing lipid in the feces of rats fed oleic acid. The fact that calcium, phosphorus, and fatty acid have the same R_f on paper chromatograms and the demonstration that a substance having the same properties as that in the fecal extracts can be prepared by known chemical methods, are strong evidence in favor of the proposed structure. Inability to purify the substance to constant composition can probably be explained on the basis of ionization of the salt as suggested by Swell *et al.*

It is also clear from the present work that complex salts may be formed biologically from eicosenoic, erucic, and linoleic acids in addition to oleic acid. In confirmation of earlier studies, no evidence was found to suggest that they are formed from saturated fatty acids. It has been possible, however, to prepare chemically a complex salt containing palmitic acid. Perhaps the higher melting point of saturated fatty acids and their lower solubility help to prevent them from forming complexes in vivo.

The biological significance of these complex salts is unknown but it appears that they may decrease the digestibility of fatty acids (3–5). In rats fed free oleic or erucic acid, the presence of complexes as high as the middle third of the small intestine has been indicated by paper chromatography. Clément, Clément-Champougny, and Raulin (12) have observed the presence of phosphorus-containing lipid in the small intestine of rats fed a diet containing 15% free fatty acids and 3% cholesterol and the lipid also contained calcium when the rats had in addition a biliary fistula (13). Neither phosphorus nor calcium was found when normal rats were fed the corresponding triglycerides with cholesterol.

The paper chromatographic methods which have been described may be useful for further studies of the occurrence and distribution of complex salts containing fatty acids. The high solubility of these complex salts in lipid solvents and the fact that they contain calcium as well as phosphate suggests the desirability of further studies on their possible role in biological systems.

Acknowledgment

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THE GROWTH-RETARDING EFFECT OF ERUCIC ACID AND ITS RELATION TO REQUIREMENTS FOR FAT-SOLUBLE VITAMINS[1]

K. K. CARROLL

Abstract

Young male rats fed a synthetic diet containing erucic acid but lacking fat-soluble vitamins plateau at a lower weight than those fed similar diets containing no fat or other individual fatty acids. Growth resumes in the erucic acid fed rats when vitamin A acetate and corn oil are administered, but neither is effective alone. The corn oil can be replaced by methyl linoleate. Vitamin A acetate alone causes some additional growth in rats plateaued on diets containing no fat or palmitic acid but it is more effective when combined with corn oil. Rats fed an erucic acid diet containing fat-soluble vitamins still plateaued at a lower weight than rats fed a similar diet containing oleic acid.

Introduction

Studies in a number of laboratories have shown that rapeseed oil has a retarding effect on the growth of rats (1–6), and the work of Thomasson and Boldingh has demonstrated that this effect of rapeseed oil is due to its high content of erucic acid (7). The growth-depressing effect of erucic acid has also been observed in our laboratory in rats fed a synthetic diet (8) but rats fed a chow diet containing erucic acid grew as well as their controls (9). In other studies, Middleton and Campbell found that rapeseed oil did not depress the growth of rats on a restricted diet relative to controls (10), and Deuel, Cheng, and Morehouse found no weight loss in adult female rats fed rapeseed oil (11). The present experiments were carried out in an attempt to reconcile these results and to obtain information on the mechanisms by which rapeseed oil and erucic acid retard growth.

Experimental

Feeding experiments were carried out with young male rats of the Sprague–Dawley strain, caged in groups of three. The animals were received in the laboratory 7 to 10 days before the start of the experiments and during this interval they were fed a stock powdered diet (Master Meal).[2] They were changed to synthetic diets at 4–5 weeks of age when they weighed 70–90 g.

The composition of the synthetic diets expressed as parts by weight was as follows: fat-free diet—casein 18, dextrose 72, salt mixture 4, and celluflour 5; fat-containing diets—casein 22, dextrose 52, fatty acid 15, salt mixture 5, and celluflour 5. A vitamin mixture was prepared by dissolving 50 mg of thiamine hydrochloride, 50 mg of pyridoxine hydrochloride, 2 mg of biotin, 10 g of choline, and 1 g of inositol in 30 ml of water, then adding 100 mg of riboflavin, 500 mg of nicotinic acid, 590 mg of calcium pantothenate, and 5 mg of folic

[1]Manuscript received December 22, 1958.
Contribution from Collip Medical Research Laboratory, University of Western Ontario, London, Ontario.
[2]Master Fox Breeder Starter Ration, Toronto Elevators Ltd., Toronto.

acid to 50 ml of alcohol. The two solutions were mixed, made to 100 ml with water and 15 ml added per kg of diet. Fat-soluble vitamins were added as indicated in the different experiments. The casein was increased in the fat-containing diets in order to keep constant the proportion of calories derived from protein but this ideal was probably not attained because of differences in digestibility of the fatty acids fed.

The sources of the materials were as follows: casein (purified)—Nutritional Biochemicals, Cleveland, Ohio; dextrose—Ingram and Bell Ltd., Toronto; Phillips–Hart salt mixture (12), Nutritional Biochemicals; celluflour—Chicago Dietetic Supply House, Inc., Chicago, Ill. Erucic acid was obtained from the Archer-Daniels-Midland Co., 2191 West 110th Street, Cleveland 2, Ohio, and was purified by two recrystallizations from three volumes of methanol at 0° C.[3] Other fatty acids were obtained from Nutritional Biochemicals, Cleveland, and the oleic acid was distilled *in vacuo* before use. Highly purified methyl oleate and methyl linoleate were obtained from the Hormel Institute, Austin, Minnesota. Cod liver oil was obtained from Ingram and Bell, Ltd., Toronto, and contained 2000 i.u. of vitamin A and 85 i.u. of vitamin D per g minimum. The corn oil (Mazola) was a commercial product of the Canada Starch Co. Ltd., Montreal and Toronto. Wheat germ oil, vitamins A, D, and K, and α-tocopherol were obtained from Nutritional Biochemicals, Ltd., Cleveland. Vitamin A was supplied as the acetate in a preparation diluted with cottonseed oil to contain one million units per gram. Vitamin D was supplied as viosterol (irradiated ergosterol) containing 400,000 U.S.P. units per g and vitamin K as menadione (2 methyl-naphthoquinone). Tocopherol was supplied as DL-α-tocopherol acetate.

Results

Previous studies in this laboratory were concerned with the effects of fatty acids on cholesterol metabolism and involved feeding experiments in which young male rats were maintained on synthetic diets for periods of 3 to 4 weeks. At the end of this time the animals on diets containing erucic acid invariably weighed less than those fed other fatty acids or a fat-free diet (8). The rats were given no fat-soluble vitamins in these experiments because the test periods were of short duration and it was not realized at the time that they were actually reaching a weight plateau after 3 to 4 weeks on the diets (Fig. 1).

If they were allowed to continue on these diets without fat-soluble vitamins, they maintained a more or less constant weight for another 4 to 6 weeks, then lost weight rapidly and soon died. If, on the other hand, a mixture of fat-soluble vitamins was added to each diet at the end of 4 weeks, the rats continued to grow, or resumed growth if they had already begun to plateau in weight. At the same time, they increased their food intake appreciably (Fig. 2).

It is interesting to note that the group of rats fed erucic acid showed a growth response in this experiment although in the absence of fat-soluble

[3]Recrystallization of the erucic acid was performed by Canada Packers, Ltd., Toronto, through the courtesy of Dr. L. J. Rubin.

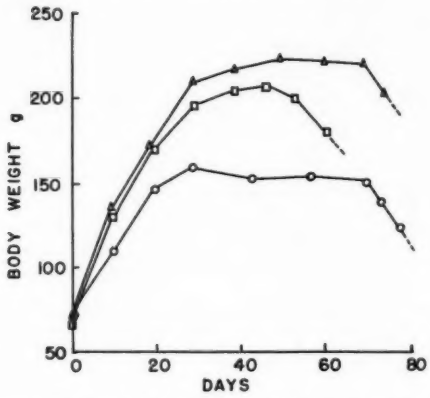

FIG. 1. Growth curves for rats fed diets containing no fat-soluble vitamins:
△ diet containing no fat,
□ diet containing 15% stearic acid,
○ diet containing 15% erucic acid.
Each curve represents an average for three rats.

vitamins they invariably plateaued 50–75 g in weight below the other experimental groups. This suggested the possibility that the growth-retarding effect of erucic acid might be related to an increased requirement for fat-soluble vitamins. Further experiments were therefore carried out to test this possibility and to study the effects of the fat-soluble vitamins individually and in different combinations.

Control studies were first carried out to determine the vitamin requirements for a growth response on a fat-free diet. Young male rats weighing 70–90 g were placed on the synthetic fat-free diet lacking in fat-soluble vitamins but otherwise adequate for the known requirements of the rat. They were allowed to reach a weight plateau similar to that shown in Fig. 1 and when their weight had remained constant for at least 10 days, various combinations of fat-soluble vitamins were added to the diet and the growth response was observed in each case. Results of some experiments are shown in Fig. 3. The best growth response was obtained with a combination of vitamin A acetate and corn oil. Vitamin A acetate alone caused some additional growth but corn oil alone had little effect. A mixture of vitamins D, E, and K gave only a slight increase in body weight.

A few control experiments were also carried out using a basic diet containing 15% palmitic acid. Rats on this diet plateaued at about the same weight as those on the fat-free diet. The growth responses obtained with vitamin A acetate plus corn oil and with vitamin A acetate alone were similar to those obtained when a fat-free diet was used.

Further experiments were then carried out using a basic diet containing 15% erucic acid. In these the animals reached a weight plateau at 150 to 160 g rather than at 200 to 225 g as in the experiments with fat-free and palmitic acid diets, and they required a shorter time to reach a weight plateau. When

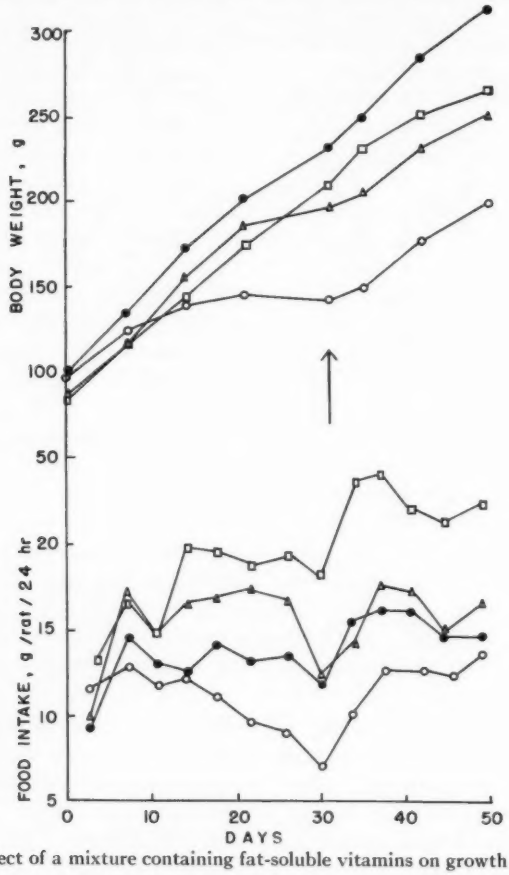

FIG. 2. Effect of a mixture containing fat-soluble vitamins on growth and food intake:

● diet containing 15% oleic acid,
□ diet containing 15% stearic acid,
△ diet containing no fat,
○ diet containing 15% erucic acid.

At the point indicated by the arrow, a mixture consisting of 5 g of cod liver oil, 5 g of wheat germ oil, and 50 mg of vitamin K in 10 g of corn oil was added at the rate of 15 ml per kg to each diet.

The points on the food intake curves represent averages for 3- to 5-day intervals for each group of 3 rats.

their weight had remained constant for at least 10 days, various fat-soluble vitamin combinations were added as in the control experiments. With this diet, the best growth response was again obtained with a combination of vitamin A acetate and corn oil. No effect was observed with vitamin A acetate alone but there was a small increase in body weight with corn oil alone (Fig. 4). A mixture of vitamins D, E, and K was ineffective, and, in experiments not shown, the addition of vitamin A acetate or corn oil to this mixture failed to produce additional growth.

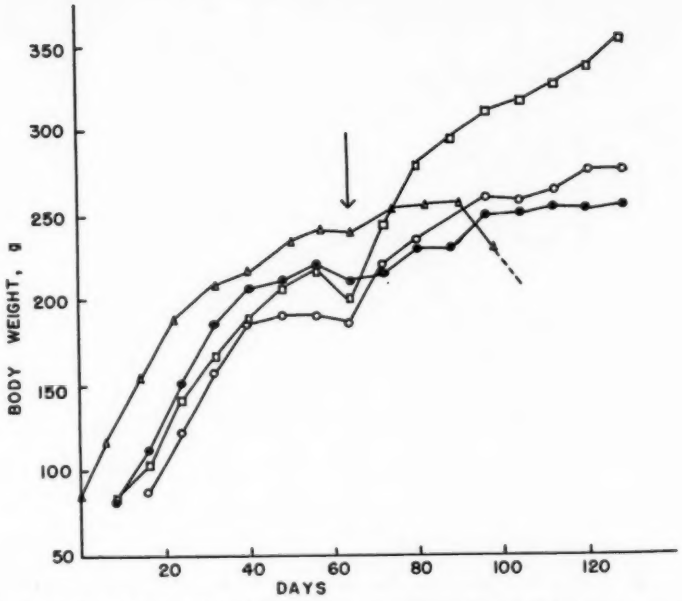

Fig. 3. Effect of fat soluble vitamin mixtures on growth of rats fed a diet containing no other fat. Fat soluble vitamin supplements were added at the point indicated by the arrow as follows:

□ vitamin A acetate (20 mg/kg) and corn oil (15 ml/kg of diet),
△ corn oil (15 ml/kg of diet),
○ vitamin A acetate (20 mg/kg of diet),
● vitamin D (5.5 mg/kg), DL-α-tocopherol acetate (110 mg/kg), and vitamin K (37.5 mg/kg of diet).

Each curve represents an average for three rats.

In order to determine whether the growth-promoting effect of corn oil in combination with vitamin A acetate was due to its content of linoleic acid, rats were fed a diet containing 15% erucic acid and also vitamin A acetate until they reached a plateau in weight. They were then given 400 mg twice weekly of corn oil, methyl linoleate, or methyl oleate by mouth and the effects on growth observed (Fig. 5). It may be noted that the response to methyl linoleate was similar to that given by corn oil whereas methyl oleate was completely ineffective. In other experiments it was shown that palmitic acid or caprylic acid in combination with vitamin A acetate were also ineffective in promoting growth of rats fed erucic acid. It appears from these results that the observed effects of corn oil are due to its content of essential fatty acids.

It was noted in the introduction that when rats were fed erucic acid mixed with a chow diet they grew as well as their controls fed the chow diet alone. It therefore seemed of interest to determine whether one could obtain optimal growth on a synthetic diet containing erucic acid by adding fat-soluble vitamins from the beginning of the experiment. The results of one such experiment are shown in Fig. 6. It can be seen that in spite of the added fat-soluble

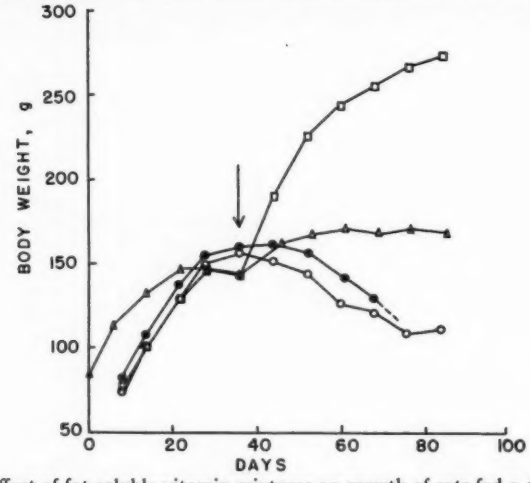

FIG. 4. Effect of fat-soluble vitamin mixtures on growth of rats fed a diet containing 15% erucic acid.

Fat-soluble vitamin supplements were added at the point indicated by the arrow as follows: □ vitamin A acetate (20 mg/kg) and corn oil (15 ml/kg of diet),
△ corn oil (15 ml/kg of diet),
○ vitamin A acetate (20 mg/kg of diet),
● vitamin D (5.50 mg/kg), DL-α-tocopherol acetate (110 mg/kg), and vitamin K (37.5 mg/kg of diet).
Each curve represents an average for three rats.

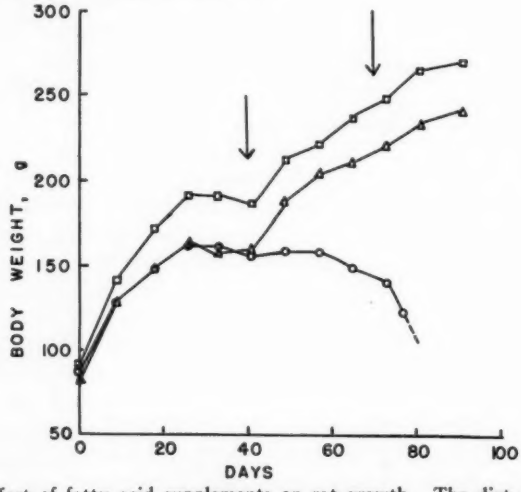

FIG. 5. Effect of fatty acid supplements on rat growth. The diet contained 15% erucic acid and also vitamin A acetate (20 mg/kg). When the rats reached a plateau in weight, supplements of fatty acids were administered by mouth, commencing at the point indicated by the first arrow and ending at the point indicated by the second arrow. The supplements were given as follows:
□ corn oil (400 mg twice weekly),
△ methyl linoleate (400 mg twice weekly),
○ methyl oleate (400 mg twice weekly).
Each curve represents an average for three rats.

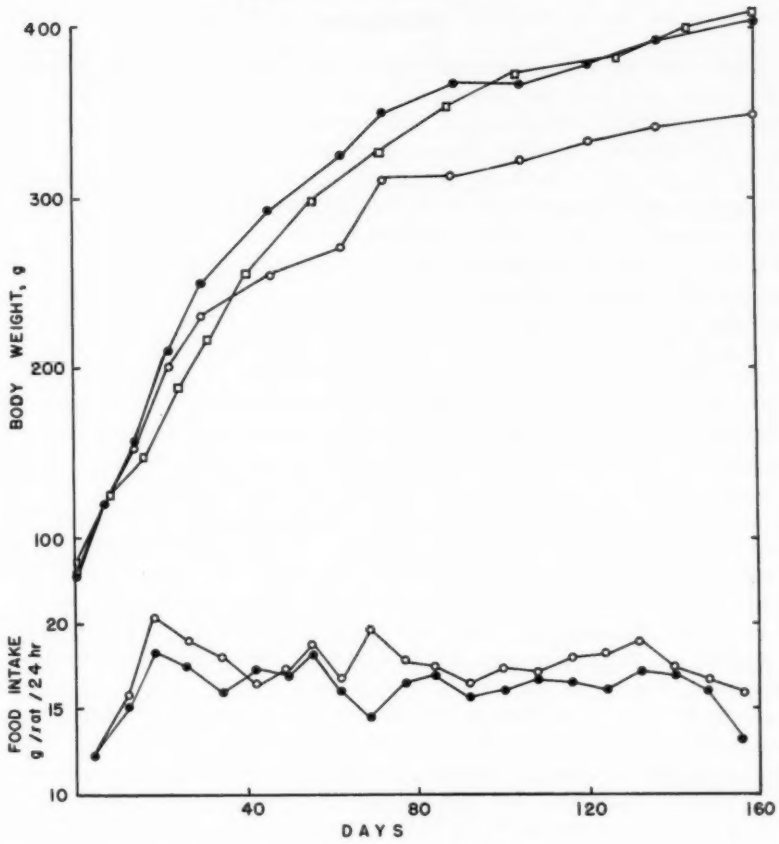

FIG. 6. Growth of rats on diets containing fat-soluble vitamins.
□ control group fed Master Meal,
● synthetic diet containing 15% oleic acid,
○ synthetic diet containing 15% erucic acid.
The synthetic diets contained vitamin A acetate (20 mg/kg), vitamin D (5.5 mg/kg), DL-α-tocopherol acetate (110 mg/kg), vitamin K (37.5 mg/kg), and corn oil (15 ml/kg). The body weight curves are averages for six rats. The points on the food intake curve represent averages for 6–8 day periods. Food intake was not measured for the group fed Master Meal.

vitamins, the group fed erucic acid did not grow as well as the control groups on chow diet or on a synthetic diet containing oleic acid. It is possible, however, that higher levels of fat-soluble vitamins may improve the growth performance of rats fed erucic acid. The food intake of rats fed the erucic acid diet was higher than that of those fed the oleic acid diet in this experiment (Fig. 6), but this does not necessarily mean a difference in food efficiency since erucic acid has a lower coefficient of digestibility than oleic acid.

Discussion

From the results that have been presented, it appears that the addition of 15% erucic acid to a diet lacking in fat and fat-soluble vitamins hastens the onset of a deficiency of vitamin A and essential fatty acids in the young male rat. It is not clear whether the apparent deficiencies of these substances are related in any way. Both are apparently required to initiate a good growth response in rats plateaued on an erucic acid diet (Fig. 4) whereas vitamin A alone appears to give a partial response in rats plateaued on a fat-free diet (Fig. 3) or a diet containing 15% palmitic acid. It seems possible that both components become deficient at approximately the same time on the erucic acid diet while the vitamin A deficiency occurs first on the other diets.

In considering the mechanism by which erucic acid may produce these effects, it has seemed unlikely that its low coefficient of digestibility could be primarily responsible since in these experiments growth was not appreciably retarded by palmitic and stearic acids, both of which have lower coefficients of digestibility than erucic acid (13). The growth retardation caused by feeding rapeseed oil can be correlated for the most part with a reduced food intake (4, 6, 10) and Thomasson has suggested that this in turn may be due to the slow rate of absorption of rapeseed oil (14). In the present experiments with erucic acid, weight gain was found to be related to food intake but the food intake was influenced by the supply of vitamin A and essential fatty acids.

This raises the question whether the effect of rapeseed oil on growth may be dependent on the availability of vitamins since this effect of rapeseed oil is apparently due to its high erucic acid content (7). Rapeseed oil contains appreciable amounts of linoleic acid (15) so that any extension of the present results to rapeseed oil would focus attention on vitamin A. However, factors other than the fat-soluble vitamins may be involved in the growth-retarding effect since it was found that rats fed erucic acid in a synthetic diet together with generous amounts of fat-soluble vitamins still reached a lower weight plateau than control groups (Fig. 6).

Studies in a number of laboratories have shown that certain other types of dietary fat may accentuate the signs of essential fatty acid deficiency in the rat. Deuel *et al.* (16) confirmed earlier findings that addition of hydrogenated coconut oil to a fat-free diet caused rats to plateau at a lower weight and in a shorter time interval than if they were fed the fat-free diet alone. A level of 30% of the oil was required to demonstrate the effect in male rats and it could be completely prevented by supplementation with linoleate. Very interesting and comprehensive experiments of a similar nature have been carried out more recently by Aaes-Jorgensen, Funch, Engel, and Dam (17–20). Aaes-Jorgensen (17) found that hydrogenated herring oil, whale oil, seal oil, and coconut oil all decreased the growth rate of rats when fed at the 28% level. In later experiments with hydrogenated arachis oil, Aaes-Jorgensen *et al.* (18) showed that the growth-retarding effects could be prevented by administration of linoleic acid.

Thomasson has also observed an effect of saturated fat on essential fatty acid deficiency using a different experimental procedure (21). Rats were first maintained on a fat-free diet and a restricted water intake until they were depleted of essential fatty acids. They were then given the test fatty acids daily in single doses of 180 mg by mouth and their subsequent weight changes were compared with those of control rats receiving no fatty acid. In these experiments myristic acid, palmitic acid, stearic acid, erucic acid, and a mixture of isomers of oleic acid all decreased body weight relative to that of the control rats.

The experimental evidence suggests that the growth retarding effects of hydrogenated fats and of erucic acid may be very similar in nature. In each case linoleic acid acts to prevent the effect when the diet is also supplemented with vitamin A. It may also be noted that rats fed hydrogenated fats develop testicular lesions with failure of spermatogenesis and very similar lesions have been found in rats fed erucic acid (9, 19). The adverse effects of hydrogenated fats on growth are apparently not due to the presence of conjugated polyenoic acids formed during the hydrogenation (22).

It is not clear from our experiments whether there is any relationship between the increased requirement for vitamin A and essential fatty acids and the increased net production of cholesterol in rats fed erucic acid (8) but work in other laboratories has suggested a relationship between vitamin A, essential fatty acids, and cholesterol metabolism. Kantiengar and Morton (23) and Green, Horner, Lowe, and Morton (24) found that dietary cholesterol lowered the liver vitamin A stores of male though not of female rats. They suggest that this may be due to more rapid utilization of the vitamin or to competition of absorbed cholesterol with vitamin A for storage in the liver. The work of Alfin-Slater *et al.* (25) and Deuel *et al.* (13) has shown alterations in cholesterol levels in rats suffering from essential fatty acid deficiency, while Peifer and Holman (26) have found that an essential fatty acid deficiency is accelerated by feeding cholesterol.

Acknowledgments

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ON THE INDUCED SYNTHESIS OF AN ENZYME REQUIRED FOR BIOSYNTHESIS OF AN ESSENTIAL METABOLITE: INDUCED KYNURENINASE SYNTHESIS IN NEUROSPORA CRASSA[1]

S. D. WAINWRIGHT[2] AND D. M. BONNER

Abstract

Brief incubation of washed mycelial pads of *Neurospora crassa* with L-tryptophan or L-kynurenine caused a marked and rapid increase in kynureninase activity. Some of the factors affecting the extent of enzyme formation were studied. Tryptophan was almost as effective an inducer of kynureninase formation as kynurenine. The significance of induced kynureninase formation as a regulator of niacin biosynthesis is discussed.

Introduction

Jakoby and Bonner (1, 2) found that extracts from *Neurospora crassa* grown in the presence of L-kynurenine or its precursor L-tryptophan had considerably higher kynureninase activities than similar extracts from mycelia grown in minimal medium.

The kynureninase enzyme catalyzes an essential step in the biosynthesis of niacin by *N. crassa*; namely, the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid (3). Formation of active kynureninase enzyme would therefore seem to be an essential condition for continued growth in minimal medium. Consequently, further study of the adaptive response reported by Jakoby and Bonner (1, 2) seemed desirable.

This communication reports some of the requirements for development of increased kynureninase activity.

Methods

All experiments were made with washed mycelial pads of the 74 A strain of *Neurospora crassa* grown on a minimal medium (4) containing ammonium tartrate 5 g, NH_4Cl 1 g, KH_2PO_4 3 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.1 g, $CaCl_2$ 0.1 g, biotin 0.5 μg, trace elements (4), sucrose 10 g, and glycerol 10 ml per liter. The initial pH was adjusted to 6.0.

Inocula were prepared from cultures grown on agar slants of 10-ml volumes of the above medium solidified with 2% (w/v) agar in 16×150 mm test tubes. Tubes were inoculated with 0.05-ml volumes of a conidial suspension prepared by the method given here. They were incubated at 30° for 4 days, transferred to room temperature to induce conidiation, and used to prepare inocula after further incubation for 5–8 days at room temperature. The conidia were suspended in 20 ml of sterile distilled water by rubbing gently with a glass rod and the suspension was filtered through sterile glass wool.

[1]Manuscript received January 10, 1959.
Contribution from Department of Microbiology, Yale University, New Haven, Connecticut.
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The minimal medium was distributed in 100-ml volumes into 500-ml Erlenmeyer flasks which were inoculated with 0.1-ml volumes of fresh conidial suspension and incubated at 30° C for 3 days.

Individual mycelial pads were removed from the cultures, thoroughly washed with distilled water, carefully blotted as dry as possible, and transferred to 125-ml Erlenmeyer flasks containing 25-ml volumes of appropriate medium.

Unless otherwise stated, the flasks were incubated at 30° C on a shaking apparatus for 4 hours. The individual mycelial pads were then thoroughly washed with distilled water, squeezed as dry as possible between sheets of filter paper, frozen at −15° C, and dried overnight on a freeze-drying apparatus.

Each dried mycelium (weighing approximately 0.2 g) was ground to a powder and suspended in 3 ml of 0.1 *M* sodium potassium phosphate buffer (pH 8.1) (1). After they had been left to stand at 4° C for 2 hours the suspensions were centrifuged at 25,000 *g* for 15 minutes, and the rather bulky debris discarded without re-extraction.* Control experiments showed that the supernatant fractions obtained usually contained approximately 60% (though sometimes more) of the kynureninase activity present in the complete suspension. However, the actual proportions present in the supernatants were remarkably uniform within a given series of mycelia (e.g. for one series the proportions were, respectively, 57, 65, 59, and 62%).

Kynureninase enzyme activities were determined by the method of Jakoby and Bonner (1) and protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (5), with serum albumin (Armour Fraction 5) as a standard. The unit of specific activity is defined as the number of micromoles of anthranilic acid formed from L-kynurenine per mg of protein per hour. Total activities are recorded as micromoles of anthranilic acid per hour per ml of extract.

In the early stages of this work L-kynurenine was obtained as the sulphate by hydrolysis of N^{α}-acetyl-L-kynurenine recovered from culture filtrates of a *Neurospora* mutant (6). When it became available, L-kynurenine sulphate was purchased from the California Foundation for Biochemical Research.† Tris-(hydroxymethyl)-aminomethane, referred to as Tris, was purchased from the Sigma Chemical Company.

Results

When washed mycelia were incubated in minimal medium supplemented with 2×10^{-3} *M* L-tryptophan there was a marked and relatively rapid rise in kynureninase activity (Fig. 1, curve A). Both the total kynureninase activity extracted and the specific activity of the extract prepared reached maximal values within 4–6 hours. Accordingly, for convenience, a standard incubation period of 4 hours was adopted for further experiments.

The influence of the L-tryptophan concentration upon the levels of kynureninase activities developed is illustrated by Fig. 2. Although there were slight

*The dilution of enzymic activity resulting from re-extraction of mycelial debris would have precluded accurate assay of extracts of very low activity.

†Now the Californian Corporation for Biochemical Research.

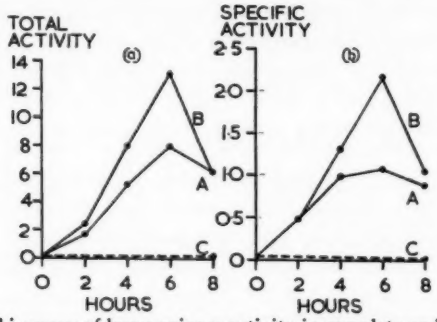

FIG. 1. Induced increase of kynureninase activity in complete and simplified medium.
(*a*) Total kynureninase activity of extract: μmoles/hr/ml.
(*b*) Specific kynureninase activity of extract: μmoles/hr/mg.
Curve A. Washed mycelia incubated at 30° C in complete minimal medium supplemented with 2×10^{-3} *M* L-tryptophan (pH 6.0).
Curve B. Washed mycelia incubated at 30° C in 4×10^{-3} *M* Tris HCl, 1.84×10^{-2} *M* NH_4Cl, 2×10^{-3} *M* $MgSO_4$, 9.1×10^{-4} *M* $CaCl_2$, 1.37×10^{-1} *M* glycerol, and 2×10^{-3} *M* L-tryptophan (pH 6.0).
Curve C. Non-induced controls in complete and simplified media.

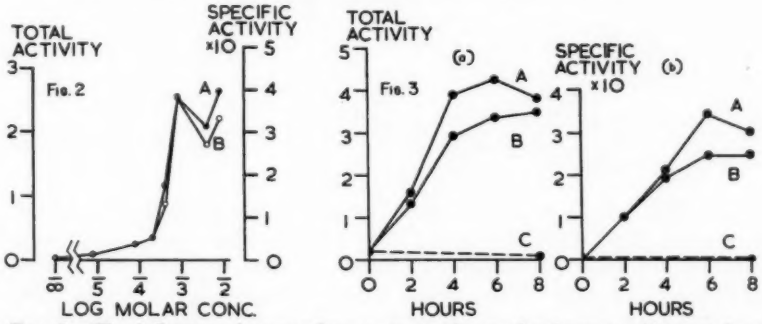

FIG. 2. The influence of tryptophan concentration on development of kynureninase activity in complete medium.
Washed mycelia were incubated at 30° C for 4 hours in complete minimal medium supplemented with various concentrations of L-tryptophan (pH 6.0).
Curve A. Total kynureninase activities, μmoles/hr/ml.
Curve B. Specific kynureninase activities, μmoles/hr/mg.

FIG. 3. The influence of casein hydrolyzate on the induced formation of kynureninase.
(*a*) Total kynureninase activity of extract: μmoles/hr/ml.
(*b*) Specific kynureninase activity of extract: μmoles/hr/mg.
Curve A. Without casein hydrolyzate.
Curve B. With 0.25% casein hydrolyzate.
Curve C. Non-induced controls.
Washed mycelia were incubated at 30° C in 4×10^{-3} *M* Tris HCl, 1.84×10^{-2} *M* NH_4Cl, 2×10^{-3} *M* $MgSO_4$, 9.1×10^{-3} *M* L-tryptophan with or without 0.25% (w/v) casein hydrolyzate (pH 6.0). Controls were incubated in similar media lacking tryptophan.

variations between the experiments, usually both the total and specific activities were maximal in extracts from mycelia incubated with 2×10^{-3} *M* L-tryptophan. Sometimes the specific activity (but not the total activity) was equally high in extracts from mycelia incubated with 4×10^{-4} *M* L-tryptophan.

Suboptimal concentrations of L-tryptophan were twice as effective as equimolar concentrations of the DL-racemic mixture, indicating that the

D-isomer was totally inactive as an inducer of kynureninase enzyme formation.

The rates and extents of increase in kynureninase formation induced by incubation with 2×10^{-3} *M* L-tryptophan for 4 hours varied with temperature, in the following order $30°>35°>25°>20°$. There was little increase in kynureninase activity when the mycelia were incubated in stationary aerobic cultures.

Experiments were then performed to determine which components of the minimal medium were required for development of increased kynureninase activity. When present in the same concentrations as in complete medium, a mixture (at pH 6.0) of potassium phosphate buffer, ammonium chloride, calcium chloride, and either glycerol or sucrose was somewhat more effective than the complete medium (Table I).

TABLE I

Replacement of complete medium by simplified phosphate medium

Medium	Total kynureninase activity of extract: μmoles/ml/hr			
	Expt. No.			
	1	2	3	4
(*a*) Complete	8.0	4.6	6.3	5.2
(*b*) Simplified	8.2	6.3	7.2	7.3

NOTE: Washed mycelia were incubated at 30° for 4 hours in either:
(*a*) Complete minimal medium (see methods) supplemented with 2×10^{-3} *M* L-tryptophan (pH 6.0), or
(*b*) 7.3×10^{-3} *M* potassium phosphate buffer, 1.84×10^{-2} *M* NH_4Cl, 9.1×10^{-4} *M* $CaCl_2$, 1.37×10^{-1} *M* glycerol, and 2×10^{-3} *M* L-tryptophan (pH 6.0).

While conducting a survey of the effects of varying both the nature and pH value of the buffer used, we found that the phosphate could apparently be satisfactorily replaced by a 4×10^{-3} *M* solution of Tris adjusted to pH 6.0 with hydrochloric acid. The highest total and specific kynureninase activities were obtained with mycelia incubated in media of pH values 5.5 to 6.0. Other variations of buffer composition (phosphate, acetate, and citrate) and pH value (range 4.5 to 6.5) tested were not satisfactory, and Tris buffer of pH 6.0 was used in place of the phosphate.

As shown in Fig. 1, the levels both of total kynureninase activity extracted and of specific activity of the extract tended to be somewhat higher for mycelia incubated with 2×10^{-3} *M* L-tryptophan in the simple Tris medium than when in the complete minimal medium (4) (Fig. 1). However, on further investigation it was found that the addition of 2×10^{-3} *M* magnesium sulphate occasionally enhanced the development of kynureninase activity. Accordingly, for all further experiments we used a medium containing 4×10^{-3} *M* Tris (pH 6.0), 1.84×10^{-2} *M* (0.1% w/v) ammonium chloride, 2×10^{-3} *M* magnesium sulphate, 9.1×10^{-4} *M* (0.01% w/v) calcium chloride, and 1.37×10^{-1} *M* (1% v/v) glycerol. The major features of the process of increase in kynureninase activity in this simplified Tris medium were essentially the same as with mycelia incubated in complete minimal medium. Maximal total and specific kynureninase activities were obtained from mycelia incubated

with L-tryptophan for 4–6 hours (Fig. 3). Vigorous aeration was required and greater activities were obtained with mycelia incubated at 30° C than at either 25° C or 35° C. Addition to the medium of a casein hydrolyzate, as a source of preformed amino acids, caused some reduction in the development of kynureninase activity (Fig. 3).

In contrast to the results obtained with minimal medium, the response to variation in the concentration of L-tryptophan was remarkably consistent. Whereas the concentration at which maximal enzyme levels were obtained in minimal medium varied between 1 and 2×10^{-3} *M* for different experiments, with the simpler medium the optimal concentration was invariably 1.4×10^{-3} *M* (Fig. 4).

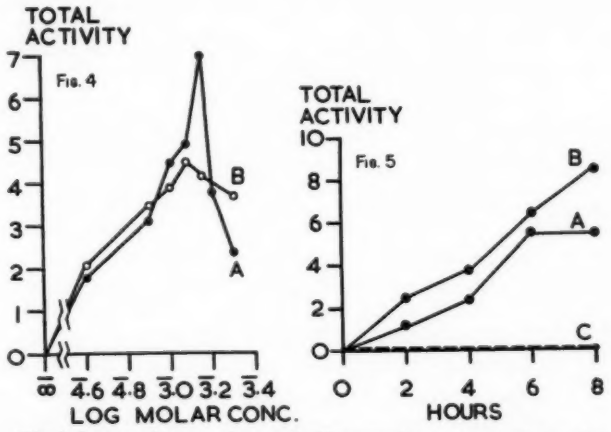

FIG. 4. The influence of tryptophan and of kynurenine concentration on kynureninase formation.
Curve A. Inducer L-tryptophan.
Curve B. Inducer L-kynurenine sulphate.
Washed mycelia were incubated (at pH 6.0) for 4 hours at 30° C in 4×10^{-3} *M* Tris HCl, 1.84×10^{-2} *M* NH_4Cl, 2×10^{-3} *M* $MgSO_4$, 9.1×10^{-4} *M* $CaCl_2$, 1.37×10^{-1} *M* glycerol, and various concentrations of L-tryptophan or L-kynurenine sulphate.

FIG. 5. Induction of kynureninase by L-tryptophan and L-kynurenine.
Curve A. Inducer L-tryptophan.
Curve B. Inducer L-kynurenine sulphate (pH 6.0).
Curve C. Non-induced controls.
Washed mycelia were incubated at 30° C in 4×10^{-3} *M* Tris HCl, 1.84×10^{-2} *M* NH_4Cl, 2×10^{-3} *M* $MgSO_4$, 9.1×10^{-4} *M* $CaCl_2$, 1.37×10^{-1} *M* glycerol with or without 1.4×10^{-4} *M* inducer (pH 6.0).

When L-kynurenine sulphate was tested as an inducer of increased kynureninase formation the concentration at which highest enzymic activities were obtained varied between 8×10^{-4} *M* and 1.4×10^{-3} *M* for different experiments. The results given in Fig. 4 were obtained in the same experiment as those for L-tryptophan.

Two major differences found between mycelia incubated with L-kynurenine sulphate and those induced with tryptophan indicate that the former substance is toxic to *N. crassa*.

First, the amount of total protein extracted from mycelia incubated for up to 4 hours with concentrations of kynurenine greater than 8×10^{-4} *M* was considerably less than that from mycelia incubated with the same concentration of L-tryptophan and usually less than from control incubated mycelia (Table II).

TABLE II

Total protein content of mycelial extracts

Expt. No.	Protein concentration (mg/ml) in extracts of mycelia incubated with: L-Tryptophan	L-Kynurenine sulphate	Control without inducer
1	7.5	5.0	5.6
2	7.9	6.6	8.1
3	9.0	8.3	7.4
4	7.7	5.8	7.1
5	6.0	4.9	5.4

NOTE: Washed mycelia were incubated at 30° for 4 hours with 4×10^{-3} *M* Tris HCl, 1.84×10^{-2} *M* NH_4Cl, 2×10^{-3} *M* $MgSO_4$, 9.1×10^{-4} *M* $CaCl_2$, 1.37×10^{-1} *M* glycerol, and 1.4×10^{-3} *M* inducer as indicated (pH 6.0).

Secondly, mycelia incubated with tryptophan attained maximal kynureninase activities within 4–6 hours, whereas mycelia treated with kynurenine sulphate did not develop maximal activities as rapidly and, indeed, continued to produce enzyme throughout the entire 8-hour period studied (Fig. 5).

It has been suggested (2) that tryptophan serves as an inducer of kynureninase formation because it is converted to kynurenine by *N. crassa*. Biosynthetic precursors of L-tryptophan might therefore also be expected to induce increased kynureninase formation, and Jakoby (2) has shown such effects of indole and anthranilic acid with the Em 5256 A strain under his growth conditions. L-Phenylalanine and L-tyrosine were also slightly active.

Under our conditions indole was very slightly active (Table III) and at the concentrations tested (2×10^{-3} *M* and 4×10^{-4} *M*) anthranilic acid caused the

TABLE III

L-Tryptophan and indole as inducers of kynureninase formation

Inducer	Concentration	Total kynureninase activities of the extracts: μmoles/ml/hr Expt. No. 1	2	3
L-Tryptophan	2×10^{-3} *M*	5.07	5.93	2.20
	4×10^{-4} *M*	1.38	1.62	1.03
	2×10^{-4} *M*	—	1.24	0.62
Indole	2×10^{-3} *M*	0.21	0.17	0.17
	4×10^{-4} *M*	0.51	0.62	0.36
	2×10^{-4} *M*	—	0.74	0.35
Control without inducer	—	0.08	0.04	0.05

NOTE: Washed mycelia were incubated at 30° C for 4 hours in 4×10^{-3} *M* Tris HCl (pH 6.0), 1.84×10^{-2} *M* NH_4Cl, 2×10^{-3} *M* $MgSO_4$, 9.1×10^{-4} *M* $CaCl_2$, 1.37×10^{-1} *M* glycerol, and inducer as indicated (pH 6.0).

total loss of the activity initially present in the untreated mycelia. L-Phenylalanine, L-tyrosine, and quinolinic acid (all at 2×10^{-3} *M*) and DL-alanine (4×10^{-3} *M*) were inactive.

The efficiencies of the major inducers of kynureninase formation found by Jakoby (2) and by ourselves are compared in Table IV.

TABLE IV

The relative efficiencies of L-kynurenine and biosynthetic precursor as inducers of kynureninase formation*

Inducer 4×10^{-4} *M*	Em 5256 A strain grown 30–40 hr with inducer†	74 A strain incubated 4 hr with inducer‡	
		Range of values	Mean value
L-Kynurenine sulphate	10	15–75	(30)
L-Tryptophan	110	10–65	(20)
Indole	65	5–15	(7)
Anthranilic acid	7	Activity lost	

*The values listed as ratios of activity present in extracts of induced mycelia to those of control non-induced mycelia.

†Data of Jakoby (2) for cultures of the Em 5256 A strain grown in complete minimal medium for 30–40 hours at 30° C.

‡Washed mycelial pads of the 74 A strain were incubated for 4 hours at 30° C in 4×10^{-2} *M* Tris HCl, 1.84×10^{-2} *M* NH_4Cl, 2×10^{-3} *M* $MgSO_4$, 9.1×10^{-4} *M* $CaCl_2$, 1.37×10^{-1} *M* glycerol, and 4×10^{-3} *M* inducer (pH 6.0).

Discussion

Brief treatment of washed mycelial pads of *N. crassa* with L-kynurenine or L-tryptophan causes a marked rise in the amount of extractable kynureninase activity.

Despite the many sources of relatively large errors in the quantitative study of this phenomenon, it seems most probable that the increase of kynureninase activity is another instance of induced enzyme biosynthesis (7). In view of the rapidity of the increase and the early attainment of maximal total activities in the presence of L-tryptophan the possibility of a more rapid growth of hyphal tips of exceptionally high kynureninase activity seems very unlikely. There is no possibility of the increase being simply one of an induced change of cell permeability, and there is nothing to indicate that the process is one of induced relief of inhibition of either enzyme formation (repression, e.g. 8, 9) or enzyme activity (inhibition by feed back, e.g. 10, 11).

Jakoby (2) has proposed that tryptophan serves as an inducer of kynureninase formation because it is converted to kynurenine by *N. crassa*. The present data are not readily compatible with this simple hypothesis. At best, kynurenine is only slightly more active as an inducer than tryptophan (Figs. 4 and 5), and the demonstrable toxicity of higher concentrations of kynurenine (Table II) does not account for the relatively low activity of small concentrations of kynurenine.

One possible interpretation of the rough equivalence of tryptophan and kynurenine as inducers of kynureninase formation is suggested by recent studies of the tryptophan synthetase enzyme reaction of *N. crassa*. Purified preparations of this enzyme catalyze the condensation of indole and serine to

form tryptophan. Yet, the intracellular substrate of tryptophan synthetase in the biosynthesis of tryptophan appears to be "bound indole" generated upon the enzyme molecule from previously bound precursor (12).

It seems entirely possible that the biosynthetic substrate of the kynureninase enzyme is "bound kynurenine" (or 3-hydroxykynurenine) formed in a similar manner. If this is indeed the case, low concentrations of "bound kynurenine" generated from tryptophan might well be as effective as much higher intracellular concentrations of free kynurenine. If the induced kynureninase activity was sufficiently high to metabolize the "bound kynurenine" more rapidly than it was formed, synthesis induced by tryptophan would cease. On the other hand, with free kynurenine the concentration of inducer would be much higher and could permit prolonged enzyme synthesis.

Regardless of the mechanism by which L-tryptophan acts as an inducer of kynureninase formation, the essential consideration would seem to be that the concentrations of inducers and precursors used in this study (Table IV) are certainly far in excess of those expected to be formed by wild-type mycelium growing in synthetic medium.

As far as we are aware, the present study provides the first demonstration of the induced synthesis of an enzyme involved in the biosynthesis of an essential metabolite.* However, we do not consider that it sheds any light on the extremely important but unresolved question of whether most, or even all, of those enzymes classed as constitutive are in fact inducible. Rather, we believe that the induced synthesis of kynureninase may be an unusual but particularly good example of the manner in which the process of induced synthesis can serve as a control mechanism for regulating the economy of an organism.

The pathway by which niacin is derived entirely from L-tryptophan (3) is given in outline in Fig. 6, together with the associated "tryptophan cycle." Two of the reactions shown are catalyzed by the kynureninase enzyme, the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid and the conversion of kynurenine to anthranilic acid (1). The former reaction is essential to the synthesis of niacin, whereas the latter forms part of the "tryptophan cycle". As kynurenine formation precedes that of 3-hydroxykynurenine, any increase in kynureninase activity would be expected to reduce the rate of formation of 3-hydroxykynurenine by diverting a greater proportion of the supply of kynurenine into the reactions of the "tryptophan cycle." Indeed, as noted above, the rapid attainment of maximal kynureninase activities with tryptophan as inducer indicate that this is probably the case. The induced synthesis of kynureninase would, therefore, seem to be of much greater significance in the operation of the "tryptophan cycle" than in the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid. We believe that the induced synthesis of kynureninase serves to regulate the intracellular levels of kynurenine by a cycle of reactions which regenerates precursor with a minimal dissipation of the energy and intermediates used in synthesis of the kynurenine. Further, the increase in potential activity of a key enzyme of the biosynthetic

*The apparent induced synthesis of acetyl ornithinase by *Escherichia coli* has now been shown to be an instance of "release" from enzyme "repression" (9).

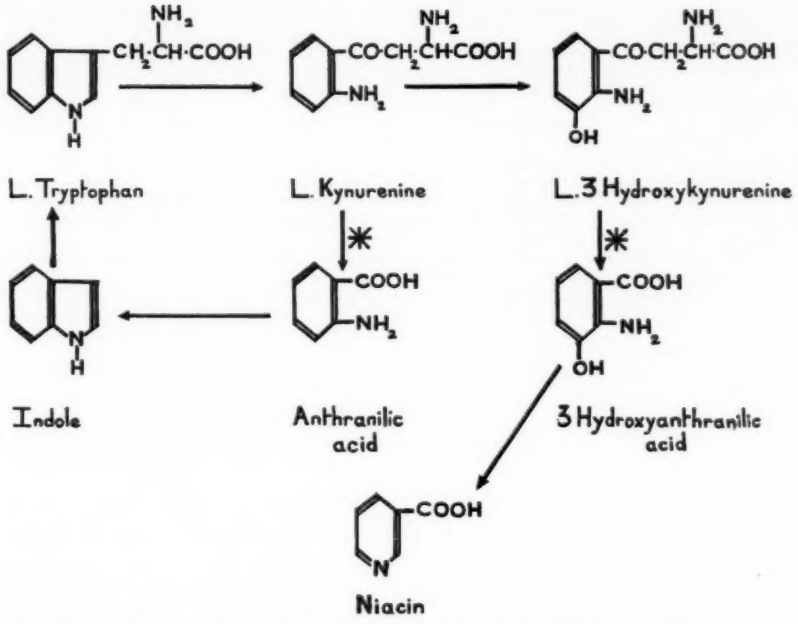

FIG. 6. Tryptophan metabolism in *N. crassa*. *Reactions catalyzed by kynureninase enzyme.

pathway would seem to be merely a fortuitous consequence of the substrate specificity of the kynureninase enzyme (1).

The rapidity of the adaptive response is of importance in another connection. Detailed studies made with other known inducible enzymes of *N. crassa* have revealed long latent periods, of up to 20 hours, prior to the onset of increased enzyme formation (13, 14). The difference is presumably related to the role of kynureninase in the formation of niacin. However, it does have an important practical consequence, namely, that it may be practicable to study the synthesis of inducible enzyme in our subcellular, enzyme-forming system derived from *N. crassa* (15, 16).

Acknowledgments

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A COMPARATIVE STUDY OF NUCLEOTIDE METABOLISM IN FISH LIVER[1]

R. J. FORREST AND R. G. HANSEN

Abstract

Anion exchange chromatography has been used to separate the acid-soluble nucleotides of the livers of several species of fish including salmon taken in fresh and salt water. This technique has produced an elution pattern of nucleotides similar to that obtained from poultry and mammalian liver. Two of the uridine-containing areas eluted from the column have been investigated in detail using enzymatic, chromatographic, and colorimetric techniques. The first of these two adjacent peaks was found to contain N-acetylglucosamine and N-acetylgalactosamine, whereas the second contained glucose and galactose. In both cases, the glucose moiety was the major constituent.

Samples of liver taken from silver salmon were fractionated and two of the enzymes acting on UDP glucose were identified. The first of these enzymes, UDPG dehydrogenase, was partially purified and the product of the reaction with UDPG was identified as UDP glucuronic acid. The second enzyme, UDPGal-4-epimerase, was separated from the dehydrogenase and characterized by enzymatic assay using UDP galactose as substrate.

Introduction

Since Caputto *et al.* (1), and Leloir (2) postulated the structure and demonstrated the coenzymatic role of UDPG* in the "galactowaldenase" conversion of galactose to glucose, this coenzyme has been isolated from a variety of sources including plants (3), chicken liver (4), mammalian liver (5), and mammary gland (4). In addition to its coenzymatic role, UDPG has been shown to function as a glycosyl donor in the formation of glycogen (6), sucrose (7), trehalose (8), and cellulose (9), and a recent report by Mills *et al.* (10) points out the central role of UDPG in the formation of glucuronides by isolated enzyme systems. The isolation of UDPG dehydrogenase from calf liver (11) and pea seedlings (12) would suggest that the direct oxidation of UDPG to UDPGA with DPN as a hydrogen acceptor is a pathway in the formation of glucuronides. However, this does not exclude the possibility of other pathways, indeed, Charalampous (13) has presented evidence for the formation of glucuronic acid from myo-inositol. A survey of the nucleotides of spring salmon liver carried out by Tsuyuki *et al.* (14) failed to demonstrate the presence of UDPG although UDPGal was found. Consideration of this apparent lack of UDPG and a high inositol content of fish liver led these authors to implicate inositol as the precursor of glucuronic acid in fish liver.

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*The following abbreviations are used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; AMP, ADP, and ATP, adenosine mono-. di-, and tri-phosphate; GMP, GDP, and GTP, guanosine mono-, di-, and tri-phosphate; UMP, UDP, UTP, uridine mono-, di-, and tri-phosphate; IMP, inosine monophosphate; UDPG, UDPGal, UDPH, UDPAH, UDPAG, UDPAGal, and UDPGA, uridine diphosphate-glucose, -galactose, -hexose, -acetylhexosamine, -acetylglucosamine, -acetylgalactosamine, and -glucuronic acid; ADPR, adenosine diphosphateribose; and ATPR, adenosine triphosphateribose.

With these results in mind and in further consideration of the possibility that fish might be different from other species tested, an investigation was undertaken to examine the nucleotides of four species of fish; namely, carp (*Cyprinus carpio*), catfish (*Ictalurus lacustris*), sockeye salmon (*Oncorhynchus nerka*), and silver salmon (*Oncorhynchus kisutch*). Of these four species the former three were taken in fresh water whereas the latter was taken in salt water. In conjunction with the nucleotide study, it has been possible to demonstrate the presence in fish liver of two of the enzymes involved in uridine diphosphateglucose metabolism. The results of these studies are the subject of this communication.

Methods

Source of Liver Samples

In all cases, the livers were excised from the fish as soon as possible after the fish were landed and immediately frozen in dry ice. The catfish liver was a composite sample obtained from seven fish of undetermined sex taken from Saginaw Bay (Lake Huron, Michigan) in June of 1958. The carp liver was excised from dying carp, taken by hand from a landlocked section of Lake Erie (near Toledo, Ohio) which had been poisoned with toxaphene approximately 20 hours previously. The sockeye salmon liver was obtained at McNary Dam on the Columbia River (Washington) on July 21, 1958. The livers from six fish (both males and females) of varying size were pooled to give 99 g of tissue from which the nucleotides were extracted. The silver salmon liver (229 g) was a composite sample from three females taken with bait on September 18, 1958, in salt water off Westport, Washington (outside of Grays Harbor).

Preparation and Chromatography of Nucleotides

Excised livers were stored in dry ice until analyzed. Without thawing, they were partially pulverized and extracted twice with 0.6 *N* $HClO_4$ (2 and 1 ml per g of tissue respectively) in a Waring blender and centrifuged. The combined supernatants were neutralized with 5 *N* KOH and left in an ice-bath overnight. This extract was then filtered and placed directly on a Dowex-1, anion exchange column (200–400 mesh) in the formate form. The chromatographic technique was the same as that reported by Hurlbert *et al.* (15) except for three minor modifications. After the nucleotides were adsorbed to the column, the column was washed with distilled water until the absorbance of the eluent at 260 mμ was below 0.1 as measured in a Beckman DU spectrophotometer with a 1-cm cell. The development of the column was initiated with 500 ml of 2 *N* formic acid followed by 1000-ml fractions of 4 *N* formic acid, 4 *N* formic acid and 0.2 *M* ammonium formate, and 4 *N* formic acid and 0.4 *M* ammonium formate. A 500-ml mixing flask was used and 15-ml fractions were collected. In all cases reported, a 1.8 $\times$ 40 cm resin bed was used (110 $\pm$ 5 ml of wet resin). The amount of nucleotide used, based on the absorbance at 260 mμ, was 300 to 400 μmole, calculated as uridine using an extinction coefficient of 10,000.

Concentration of Nucleotides

Appropriate fractions from the column (as determined by absorbance at 260 mμ) were pooled and the nucleotides were adsorbed directly on to 2–3 g of charcoal (Mallinckrodt activated NF powder) and the suspension was filtered through a Buchner funnel. The charcoal was then eluted with 100 ml of 15% pyridine and the filtrate was extracted twice with 50-ml portions of chloroform (16). The water layer was then filtered into a flask and concentrated by freeze-drying or vacuum-drying at room temperature. The residue was dissolved in water and appropriate aliquots were taken for analysis.

Analytical Methods

The determination of acetylhexosamines, the oxidation of hexosamines to pentoses, and the chromatography of acetylhexosamines, hexosamines, hexoses, and pentoses using butanol, pyridine, and water (6:4:3) were carried out as described by Pontis (16) and Reisig *et al.* (17). The UDPA hexosamines and UDP hexoses were hydrolyzed to acetylhexosamines and hexoses respectively with 0.02 *N* HCl in a boiling water bath for 30 minutes. Prior to chromatography, the acetylhexosamines, hexoses, and pentoses were deionized with Amberlite MB-3.

The enzymatic identification of UDPG and UDPGal was carried out using a highly purified UDPGal-4-epimerase fractionated from *S. fragilis* and a purified UDPG dehydrogenase from calf liver. The assay system was similar to that described by Hansen (18) and Maxwell (19). As the dehydrogenase from calf liver is specific for UDP glucose, the separate addition of dehydrogenase and epimerase permits an estimate first of UDP glucose, then, with epimerase addition, an estimate of UDP glucose formed from UDP galactose. The reaction was carried out using 0.5 μmole DPN, 0.02 ml of UDPG dehydrogenase (prepared according to Strominger *et al.* (11), 0.015–0.025 μmole UDP hexose, and 0.01 ml UDPGal-4-epimerase (5.7 mg/ml) diluted to a final volume of 0.5 ml with 0.2 *M* glycine buffer at pH 8.7. The optical density at 340 mμ of the DPNH formed was used to follow the reaction.

Results

Identification of the Hexose Portion of the Uridine Nucleotides

A typical elution pattern from the resin-separation of nucleotides in the extract of silver salmon liver and comparable chromatograms from chicken and rat liver are shown in Fig. 1. Qualitatively similar patterns were obtained from all four species of fish. The two peaks considered in detail in this communication are designated M and N and are UDPA hexosamine and UDP hexose respectively.

The results of the enzymic assay of the UDP hexose fraction are tabulated (Table I). In all four species of fish UDP glucose was the major constituent; UDPGal ranged from 14 to 26% of the total UDP hexose fraction. Similar analyses for rat and chick liver yielded 30 and 80% UDPGal, respectively. The high UDPGal value for this sample of chick liver is expected because of the presence of galactose in the diet of the chick (20). The presence of glucose

and galactose in acid hydrolyzates of the UDP hexose fraction was qualitatively confirmed by chromatography. A chromatogram comparing fish and rat liver, and the liver from galactose-fed chicks was photographed and reproduced

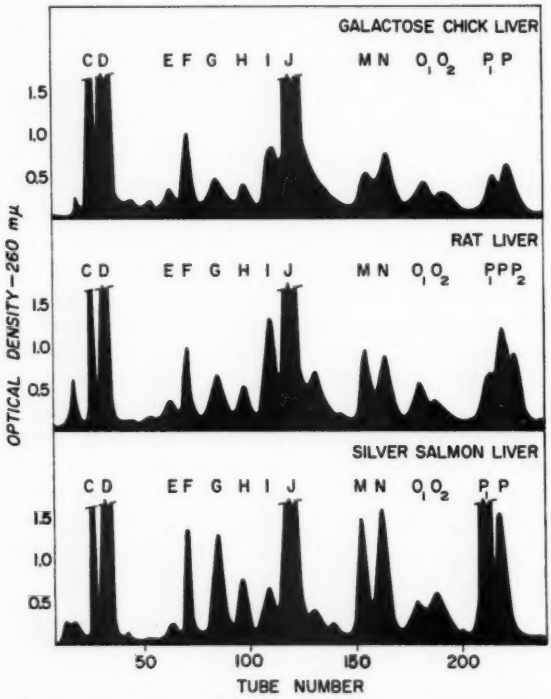
GALACTOSE CHICK LIVER
C D E F G H I J M N O1 O2 P1 P
RAT LIVER
C D E F G H I J M N O1 O2 P1 P P2
SILVER SALMON LIVER
C D E F G H I J M N O1 O2 P1 P
OPTICAL DENSITY – 260 mμ
1.5
1.0
0.5
50
100
150
200
TUBE NUMBER

FIG. 1. Ion exchange chromatography of the acid-soluble nucleotides of liver samples by the method described in the text. The major constituent in the peaks (in one or more of the liver samples) where they have been identified are as follows: (C) DPN, (D) AMP, (E) TPN, (F) GMP, (G) ADPR, (H) IMP, (I) UMP, (J) ADP, (M) UDPAH, (N) UDP hexose, (O_1) ATPR, (O_2) GDP, (P_1) UDPGA, and (P) ATP. Peak P_2 is probably UDP.

TABLE I

Enzymic analysis of UDP hexose fractions

Liver sample	μmole DPNH formed × 10^2		% UDPGal
	Without UDPGal-4-epimerase	With UDPGal-4-epimerase	
Silver salmon	1.50	1.99	24
Sockeye salmon	1.26	1.71	26
Carp	1.64	2.10	21
Catfish	1.16	1.35	14
Rat	1.45	2.07	30
Chick (galactose fed)	0.26	1.68	84

NOTE: The assay was carried out using UDP hexose, 0.5 μmole DPN, 0.2 ml UDPG dehydrogenase and 0.1 ml UDPGal-4-epimerase, and 0.2 *M*, pH 8.7 glycine buffer to 0.5 ml in a microcuvette with a 1-cm light path. This was compared to a cuvette lacking the UDPGal-4-epimerase and also a blank lacking substrate and UDPGal-4-epimerase. DPN reduction was measured at 340 mμ.

in Fig. 2. Glucose is the major normal component; however, in chicks, galactose feeding alters this. Thus the enzymic results are qualitatively confirmed.

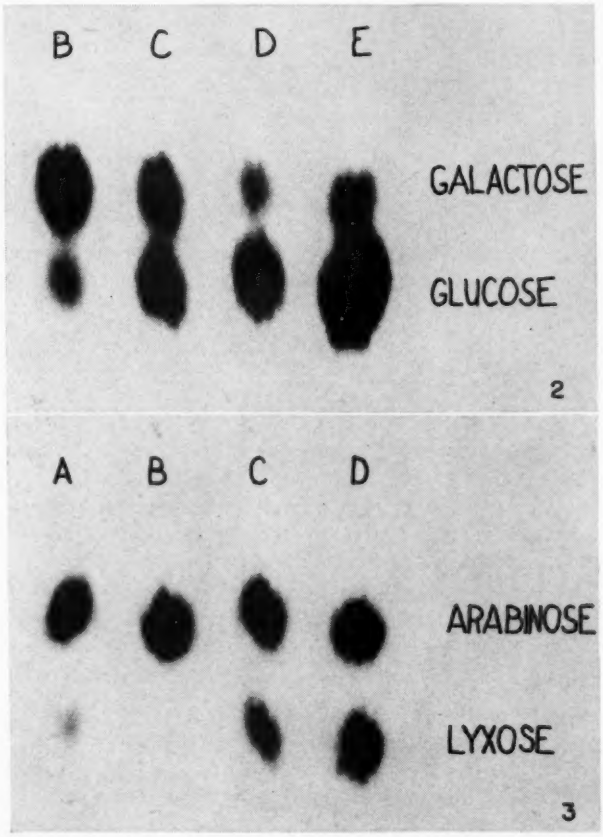

FIG. 2. Descending chromatogram of the hydrolyzed–deionized UDP hexose fractions from: (B) liver from galactose-fed chicks, (D) rat liver, and (E) silver salmon liver. Sample C is a mixture of known glucose and galactose. Chromatography was carried out with butanol, pyridine, and water (6:4:3) for 24 hours on Whatman No. 1 paper.

FIG. 3. Descending chromatogram of silver salmon liver pentoses formed after hydrolysis of UDPAH to hexosamine and conversion to pentoses, by treatment with ninhydrin in citrate buffer. The samples are: (A) silver salmon UDPAH, (B) standard UDPAG (hydrolyzed and converted to pentoses), (C) standard glucosamine and galactosamine (treated with ninhydrin), and (D) standard arabinose and lyxose. Chromatography was carried out for 24 hours in butanol, pyridine, and water (6:4:3).

The results for the colorimetric assay of the acetylhexosamines and hexosamines are tabulated (Table II). In the case of the carp liver, the absorbance at 260 mμ was abnormally high due to incomplete removal of the pyridine as this sample was concentrated on the rotary dryer. Lyophilization, employed

TABLE II

Analysis of UDPA hexosamine fractions

Liver samples	μmole/ml* UDPAH	μmole/ml acetylhexosamine	μmole/ml hexosamine	Ratio AH/Hex	% acetyl-glucosamine†
Silver salmon	3.15	2.60	3.24	0.80	73
Sockeye salmon	2.95	2.04	2.75	0.74	61
Carp	—	1.30	1.67	0.78	65
Catfish	1.90	1.16	1.61	0.72	58

*Determined by absorbance at 260 mμ after purification and concentration.
†Determined as described by Pontis (16) and Reisig *et al.* (17) based on the principle that in the acetylhexosamine assay acetylgalactosamine gives 35% of the color intensity of an equivalent amount of acetylglucosamine. The two hexosamine components, on the other hand, develop the same amount of color with the particular reagents used, thus allowing one to calculate the percentage of each constituent present in the original mixture using simultaneous equations with the two sets of data.

in all subsequent studies, tends to remove traces of pyridine from nucleotides quantitatively. This is adequately demonstrated with the uridine: acetylhexosamine: hexosamine ratios of 1.18:0.72:1, 1.07:0.74:1, and 0.97:0.80:1 for the catfish, sockeye salmon, and silver salmon liver respectively based on hexosamine as unity. The corresponding values for the percentage of acetylgalactosamine in the four species of liver ranged from 28 to 42, which is comparable to previous findings (16, 21).

Due to the limited amount of material available, acetylgalactosamine and galactosamine (as well as the glucose counterparts) were identified only in the case of the silver salmon liver using the chromatographic methods described by Pontis (16). However, positive identification of arabinose (from glucose) and lyxose (from galactose) was obtained when the hexosamines of the catfish and sockeye and silver salmon liver were oxidized to pentoses with ninhydrin (16), deionized, and chromatographed. The results obtained with an aliquot of the hexosamine hydrolyzate from silver salmon liver are reproduced in Fig. 3.

Partial Purification of UDPG dehydrogenase and UDPGal-4-epimerase

The purification of the dehydrogenase from fish liver was adapted from that reported by Strominger *et al.* (12) for calf liver. The initial precipitation of the extract with 40% ammonium sulphate as recommended for calf liver enzyme did not sharply separate the original dehydrogenase activity from C-4-epimerase and consequently this step was modified. Ten grams of an acetone powder (23) of salmon liver was extracted for 1 hour with 300 ml of water. After centrifugation, ammonium sulphate was added to 50% saturation (308 g/liter) to the supernatant. The dehydrogenase was completely precipitated (P-1) under these conditions while the epimerase remained in solution (S-1). The epimerase, free of dehydrogenase (P-2), could then be obtained by further addition of ammonium sulphate (48 g/liter) to the supernatant (S-1). The dehydrogenase was purified by dissolving P-1 in water and adding ammonium sulphate to 91 g/liter, by discarding the precipitate, and by further fractionation as described by Strominger *et al.* (11) for calf liver. The final precipitate (P-7) containing 70% of the original dehydrogenase activity was dissolved in water and stored at minus 15°.

FIG. 4. Descending chromatogram of uridine derivatives in isobutyric acid, concentrated ammonium hydroxide, and water (66:1:33) for 36 hours. Spots were located with an ultraviolet mineral light model SL 2537 lamp and traced. The samples are: (1) standard UDPG, (2) peak N (UDPG after ion exchange chromatography), (3) standard UTP, UDP, and UMP (from top to bottom), (4) enzymatically prepared UDPGA, (5) standard UDPGA (Sigma), (6) silver salmon liver UDPGA, (7) sockeye salmon liver UDPGA.

Assay for UDPGal-4-epimerase

Fraction P-2 showed no dehydrogenase activity with either UDP glucose or UDP galactose as substrate. However, with calf liver dehydrogenase a UDP glucose-dependent reduction of DPN occurred. UDP galactose would serve as substrate when fraction P-2 was added (Table III) thus establishing the presence of UDPGal-4-epimerase in the fish liver acetone powder. This reduction was approximately linear with time and proportional to added enzyme during the initial stages of the reaction (Fig. 5).

Conversion of UDPG to UDPGA by the Fish Liver Dehydrogenase

To permit isolation of the products of the reaction of the fish liver dehydrogenase with UDPG, 8 ml of P-7 were incubated at room temperature for 90 minutes with 80 mg of DPN, and 20 mg of UDPG in a final volume of 150 ml of glycine buffer (0.2 *M*, pH 8.7). At the end of the incubation period, 8 ml of 70% $HClO_4$ was added and the precipitated protein was discarded. The supernatant was neutralized with 5 *N* KOH and left in an ice-bath over-

TABLE III

Substrate dependence of the fish liver UDPGal-4-epimerase

Additions*				
Calf liver UDPG dehydrogenase (0.03 ml)	Fish liver UDPG-4-epimerase (0.01 ml)	UDPG (0.1 μmole)	UDPGal (0.1 μmole)	μmole DPNH formed in 10 min.
−	+	+		0
−	+	−	+	0
+	−	−	+	0
+	+	−	+	0.044

*As well as the listed constituents, each cuvette contained 1 μmole of DPN and glycine buffer (pH 8.7, 0.2 *M*) to 0.5 ml. Reduction of DPN was measured at 340 mμ.

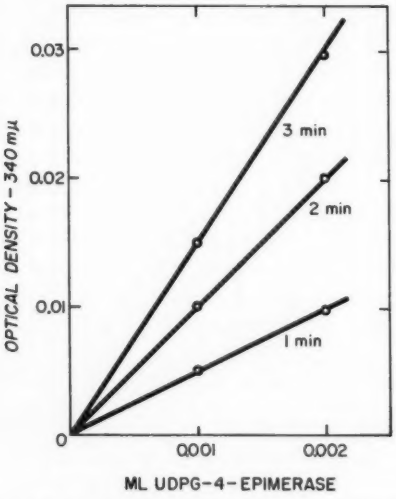

FIG. 5. Reduction of DPN with fish liver UDPGal-4-epimerase and calf liver UDPG dehydrogenase with UDPGal as substrate. Assay system consisted of 0.04 μmole UDPGal, fish liver 4-epimerase, 0.02 ml UDPG dehydrogenase, 0.5 μmole DPN, and glycine buffer (0.2 *M*, pH 8.7) to 0.5 ml in a microcuvette with a 1-cm light path. DPNH formation was measured at 340 mμ and compared to a blank without substrate.

night. The extract was then filtered and subjected to ion exchange chromatography as described above.

Four nucleotides were identified in the reaction mixture corresponding to fractions designated C, G, N, and P_1, in Fig. 1. Fractions C and N were found to be DPN and UDP glucose respectively. Fraction G, on the basis of phosphorus and ribose analysis, and by its chromatographic behavior has been identified as ADPR (24) the acid hydrolysis product of reduced DPN (25). Peak P_1 was compared with and found to be identical with an authentic sample of UDPGA (Sigma Chemical Company) and two samples of UDPGA isolated from the salmon liver samples. When chromatographed in isobutyric acid, water, and ammonium hydroxide (66:1:33) (26) all four of the above

samples of UDPGA had the same R_f value (Fig. 4). When the samples were analyzed for glucuronic acid as described by Dische (27), the enzymatically prepared UDPGA had a ratio of uridine to glucuronic acid of unity. Upon hydrolysis in 1 *N* HCl for 30 minutes at 100° C, and chromatography in butanol, pyridine, and water (6:4:3) and ethyl acetate, pyridine, and water (2:1:2), the hexose moiety had the same mobility as authentic glucuronic acid.

Quantitative data pertaining to the uridine nucleotide content of liver is sparse. Results presented in Table IV for salmon liver reveal the presence of

TABLE IV

Quantitative estimate of the uridine nucleotides of liver

	μmole/100 g wet liver†		
Liver source*	UDPAG	UDPG	UDPGA
Silver salmon	21.4	26.0	39.6
Sockeye salmon	16.8	19.7	25.7
Normal chick[21]	29.6	21.4	
Galactose-fed chick[21]	22.1	35.5	
Guinea pig[22]	20.4	21.6	

*Superscripts refer to reference number where values were obtained.
†Based on the absorbance at 260 mμ after ion exchange chromatography.

significantly high concentrations of UDPGA, namely, 39.6 and 25.7 μmole/100 g of wet tissue for silver and sockeye salmon liver respectively. Comparative quantitative data for other species of liver are not available but the marked difference in UDPGA content of fish liver and rat and chick liver is aptly demonstrated in Fig. 1 (fraction P).

Tsuyuki *et al.* were unable to detect any GTP or UTP in fish liver. In rat liver, these nucleotides have been identified and are eluted from the ion exchange column after ATP when the system of Hurlbert *et al.* (15) is employed. In the present study (in the case of the catfish and salmon liver samples), the elution was carried farther than indicated in Fig. 1 with 1 *M* ammonium formate and fractions were obtained that corresponded to UTP and GTP but these have not been further characterized.

Discussion

UDP glucose is the major component of the UDP hexose fraction of the nucleotides of livers of chickens and rats and rat mammary gland (4). This communication demonstrates that UDP glucose is also prevalent in liver UDP hexose of four species of fish. As the livers of salmon taken in fresh and salt water also contain approximately the same relative proportion of UDPG and UDPGal, the possibility of the environmental condition affecting these uridine nucleotides is eliminated. The relative proportion of UDPG and UDPGal in liver and other tissues is normally about 3 to 1, which also is the equilibrium ratio reported (28, 29) for the "galactowaldenase" enzyme which interconverts these two uridine hexoses. It would not be surprising to find that this equilibrium exists in tissue where the enzyme is present. The

present investigation also confirms the observation that this 3 to 1 ratio of UDPG to UDPGal can be reversed in chickens by feeding a 15% galactose diet (20).

The presence of high concentrations of UDPGA in salmon liver poses the question as to the means of its formation. It has been suggested (14) that inositol may function as a precursor. The present results indicate that fish liver contains UDP glucose and UDPG dehydrogenase and the partially purified enzyme forms UDPGA directly from UDPG. This does not rule out the possibility of other pathways or, for that matter, the formation of UDP glucose from inositol. However, it now seems reasonable to conclude that fish liver is similar to calf liver in that both possess a dehydrogenase system for the formation of UDPGA.

Although the ammonium sulphate fraction containing UDPGal-4-epimerase (reaction 1) was not extensively purified, it was obtained free of UDPG dehydrogenase (reaction 2).

$$\text{Reaction 1:}\quad \text{UDP galactose} \xleftrightarrow{\text{UDPGal-4-epimerase}} \text{UDP glucose}$$

$$\text{Reaction 2:}\quad \text{UDP glucose} + 2\ \text{DPN}^+ \xrightarrow{\text{UDPG dehydrogenase}} \text{UDP glucuronic acid} + 2\ \text{DPNH} + 2\ \text{H}^+$$

UDPGal could serve as substrate for reduction of DPN by the specific UDP glucose dehydrogenase when either a highly purified fraction from yeast or the UDPGal-4-epimerase from fish liver was added. This evidence, in conjunction with the 75:25, UDPG:UDPGal ratio in fish liver is indicative of the presence of UDPG-4-epimerase.

UDPAGal was identified in bovine liver by Pontis (16). It also occurs in chicken liver (20), rat mammary gland (18), and in fish liver. Thus it would appear that glucose and galactose as uridine acetylhexosamine and as uridine diphosphatehexose derivatives are normally present in the livers of fish, chickens, and mammals. The enzymes involved in uridine acetylhexosamine metabolism have not yet been adequately characterized but preliminary reports have appeared (30, 31). The presence of relatively high levels of UDPAH as well as UDP hexose and particularly UDPGA (Table IV) (Fig. 1) in salmon liver suggests that this material might be an excellent source for these enzymes.

Tsuyuki *et al.* (14) reported the absence of UDP in perchloric acid extracts of liver when columns were run at 0° C. With chromatography at room temperature employed in this study a well-defined UDP peak was not obtained. Rechromatography of the P area by the ammonium formate resin method of Hurlbert *et al.* (15) revealed that the UDP is closely associated with ATP under the conditions employed. This observation has been substantiated by others (32). However, the amount of UDP even from columns eluted at room temperature is small.

The advantages of low temperature chromatography remain to be fully assessed. There can be little doubt that lowering the temperature from 25 to 0° C will decrease the amount of hydrolysis of nucleotides. A report has

appeared (14) in which low temperature chromatography resulted in the identification of a complex adenine compound which may not have been found if the procedure had been carried out at room temperature. Denamur *et al.* (33) were able to increase markedly the yield of GDP fucose from sheeps' milk by resorting to a trichloroacetic acid extraction procedure followed by low temperature chromatography. However, there are other important factors to consider. Routine column chromatography by the authors, conducted at room temperature according to the conditions described in the text, has resulted in good recovery of the particular uridine nucleotides under consideration. Although Tsuyuki *et al.* were unable to isolate UDPG from columns eluted at 0–5° C, it is highly unlikely that UDPG was formed in the present study by chemical means as a result of the higher temperature (25° vs. 0–5°). Actually, the glycosyl phosphate bond in glucose-1-phosphate and therefore UDP glucose is more stable to acid hydrolysis than the corresponding bond in galactose-1-phosphate (34). That UDP glucose exists in the nucleotide extract in some subtly combined form or that it actually separates from UDP galactose by the conditions employed by Tsuyuki *et al.* (14) seems remote. Further study is required to clarify this point. One other possibility should be considered and that is a difference in species of fish. With respect to the uridine nucleotides, several species of fish were all similar and in a qualitative way comparable to mammals and chickens but apparently different than the species of salmon studied by Tsuyuki *et al.* In any case, the present results seem to be definitive both for the occurrence of UDP glucose as well as a dehydrogenase and a C-4-epimerase specific for UDP glucose.

Acknowledgments

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THYROXINE AND INSULIN INTERACTION[1]

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Abstract

Rate of body weight gain of both the young chick and the young rat was markedly inhibited following ingestion of amounts of thyroxine which were greatly in excess of physiological requirements. The rate of body weight gain of the young rat was also markedly inhibited in alloxan diabetes. The administration of insulin partly or largely prevented the observed effect on body weight in both conditions. The administration of thyroxine caused a reduction of rat skeletal muscle mass which was partially prevented when insulin was given concurrently with thyroxine.

Reduced body weight gain and muscle mass in the rat were accompanied by marked increases in the muscle DNA and nuclei concentration. Nuclear diameter and the amount of DNA per nucleus remained unchanged. RNA concentration of the muscle was not reduced. The beneficial effect of insulin administration on body weight and muscle mass was accompanied by a preventive effect against changes in muscle DNA and nuclei concentration. However, since the DNA content of muscle bundles remained relatively unchanged and since muscle bundle weights changed proportionately with body weight it would appear that DNA content does not reflect change in muscle mass.

The effectiveness of insulin in the thyroxine-treated animal appears to be due to an improved utilization of energy. A hypoglycemic action of thyroxine implies an antagonism between the two hormones in their mechanism of action. The relationship between the two hormones is not direct since the administration of insulin does not affect the increased plasma protein-bound iodine levels resulting from thyroxine treatment.

Introduction

The increased metabolic rate and loss of energy as heat observed in an animal treated with excess thyroxine may be accompanied by body weight loss (9). Rate of oxidation has been shown to be controlled by rate of phosphorylation in the normal animal but in the animal treated with excess thyroxine this control no longer exists and respiration is elevated dependent upon the level of thyroxine employed (14, 20, 25). The hyperthyroid rat has shown an increased rate of turnover of P^{32} in muscle and a decreased rate in kidney and liver suggesting that thyroid activity influences the rate of transfer of phosphorus across cell membranes (12).

Insulin has been shown to increase phosphate turnover and rate of phosphorylation without a change in rate of respiration (26). The suboptimal growth and protein synthesis of diabetes have been shown to be improved by the administration of insulin (17) although insulin treatment resulted only in increased fat deposition in the hypophysectomized rat (34) and in the otherwise untreated animal (3).

Some evidence exists for a relationship between thyroxine level and insulin requirement. Thyroidally active substances have been shown to increase hexokinase content (30) and insulin uptake (7) of muscle and to effect marked increases in the weight of the islets of Langerhans (16), in the β/α cell ratio of the pancreas (15) and in insulin secretion (10, 35). Severe overdosage with

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thyroxine has been shown to cause degeneration of the β cells with the appearance of diabetes (29). It would appear that an increased demand for insulin is largely met by increased pancreatic activity in the hyperthyroid state but that insufficiency of pancreatic potential can result in diabetes under the influence of excess thyroxine.

The foregoing references suggest a relationship between the functions of thyroxine and insulin in metabolism. In an effort to study some aspects of this relationship in the young, growing animal a hormonal imbalance was created either by the administration of levels of thyroxine greatly in excess of physiological requirements or by the impairment of pancreatic activity by alloxan. The experiments reported in this paper were designed to demonstrate the effect on body weight and nucleic acid of the disturbance of the above hormonal balance and the restorative action of insulin. To this end a study was made of changes in rate of body weight gain, feed utilization, and muscle nucleic acid concentration. Measurements were also made of liver glycogen, blood glucose, plasma protein-bound iodine, and certain essential muscle components.

Experimental Procedure

Test animals were young chicks of various breeds or inbred, albino weanling rats. Water and feed were given ad libitum. The chicks were fed a good quality diet calculated to contain 209 Cal/100 g and 18.2 g digestible crude protein/100 g diet (11). The diet for rats was powdered commercial fox cubes. Laevo or racemic thyroxine as the monosodium salt was incorporated in the diet by careful premixing. Protamine zinc insulin (Connaught Research Laboratories, Toronto) was administered either daily or on alternate days by subcutaneous injection and glucose was injected concurrently to prevent hypoglycemia. The ratio of glucose to insulin was kept constant at 0.34 g glucose per unit of insulin. The insulin and glucose dosages were increased by fixed increments based on body weight changes in order to keep pace with increasing thyroxine ingestion. The experimental intervals varied from 3 to 12 weeks depending on the effect of treatment on growth rate. Alloxan was administered by intraperitoneal injection of a 4% aqueous solution in the amount of 200 mg/kg body weight. Chicks failed to develop diabetes following alloxan treatment. Two weeks after the administration of alloxan to the rat, individual blood samples were analyzed for glucose content and only those rats having blood glucose values in excess of 300 mg% were selected for the trials. Environmental temperature was not accurately controlled.

Body weights were recorded at fixed intervals and the trials were terminated when a persistent inhibition of body weight gain had been established in the thyroxine-treated or diabetic animals. Venous blood samples were collected and pooled for plasma protein-bound iodine determination by the method of Barker *et al.* (2). Individual blood samples were analyzed for glucose content by the method of Mendel *et al.* (24). Liver glycogen was determined on pooled samples by the method of Loewus (19) after the animals were killed by intra-

venous injection of air. For the determination of muscle nucleic acids the hind legs of each rat were removed under deep Nembutal anaesthesia and immediately frozen in liquid air. The material was kept frozen at −20° C until analyzed. Pooled samples, comprising approximately equal portions of muscle from each leg of all animals comprising a group, were crushed in a chilled steel cylinder and then pulverized in a mortar with liquid air just prior to analyzing for these components. For these determinations, acid-soluble compounds were removed in the cold, phospholipids were removed, and the combined nucleic acids were then extracted according to the method of Schneider (28) in order to prevent the contamination with protein breakdown products which resulted when separation of the nucleic acids was attempted. However, this extract was found to contain glycogen in sufficient quantity to interfere in the determination of ribose by any of the methods based on furfural reaction (1, 4, 6, 8, 22, 23, 27, 32). Interfering polysaccharides were removed by saturation with ammonium sulphate and the ribose content of the extracts was determined by the Mejbaum orcinol reaction as outlined by Glick (13). However, it was found also that some hydrolysis of glycogen had occurred during the hot TCA extraction. The resultant small degree of hexose interference in the orcinol reaction was overcome by the application of the Brown dichromatic colorimetric procedure (4). The desoxyribose content of the extracts was determined using the Burton modification (5) of the Dische diphenylamine reaction. Desoxyribose, in the amount found in muscle tissue, had no appreciable effect on ribose estimation in the orcinol reaction. Muscle nuclei were isolated according to the method of Marshak (21) and clumping was prevented by ensuring a fixed acid pH throughout the isolation procedure. The nuclei were fixed and stained in acid medium using aceto-carmine. The nuclei concentration of each pooled treatment sample was determined by means of a haemocytometer and photomicrographic measurements were made of nuclear diameters.

Results

The data for a series of experiments which were conducted with chicks are shown in Table I. The results indicate that the prolonged administration of thyroxine in an amount which resulted in a marked elevation of the plasma protein-bound iodine level of the blood is accompanied by persistent, statistically significant decreases ($P < 0.01$ by analysis of variance) in the rate of body weight gain, in the tibia length, and in the width of the epiphyseal cartilage plate. In all cases the concomitant administration of insulin resulted in a statistically significant increase ($P < 0.01$ by analysis of variance) in rate of body weight gain compared to the thyroxine-treated group. It is seen in Fig. 1 that the administration of insulin resulted in an improved rate of gain throughout the treatment period. Because the level of insulin administered was always based on the group average weight gain for the previous week and because a slight excess of insulin was found to be lethal the body weight was not always completely restored to normal. The increase in feed intake per

TABLE I

Insulin mitigation of growth inhibition in the thyroxine-treated chick

Trial No.	Treatment	Length of treatment (wk)	Mortality, %	Av. final body wt. (g)	Av. wt. gain for final wk (g)	Av. feed intake for final wk (g)	Av. feed/gain for final wk (g/g)	Av. tibia length (mm)	Av. liver glycogen (mg/g)	PBI,* μg%
1	Control	8	5	1350	202	654	3.1	122.9	20.0±2.9	2.2±0.1
	Control + glucose		0	1399	280	749	2.8	119.5	22.3±2.2	4.3±0.1
	0.75 units insulin		10	1491xx	272	779	2.9	122.0	14.6±1.6	2.5±0.0
	0.0015% *dl*-thyroxine in diet		10	1119xx	164xx	750	4.7	118.8xx	3.5±0.1	42.0±0.9
	Thyroxine + 0.75 units insulin		5	b 1237xx	b 198ns	821	4.4	117.3xx	2.5±1.7	36.6±0.5
	Thyroxine + 1.5 units insulin		40	b 1263ns	b 288ns	956	3.4	117.0xx	4.1±1.0	46.6±2.1
2	Control	8	0	1273	148	734	5.2	120.0		2.5±0.0
	0.5 units insulin		0	1224	173	784	5.1	119.2		1.6±0.0
	0.003% *dl*-thyroxine in diet		70	692xx	90xx	962	10.7	103.7xx		29.9±0.8
	Thyroxine + 0.50 units insulin		55	b 978xx	96xx	854	10.4	108.1xx		26.7±0.6
	Thyroxine + 0.75 units insulin		40	b 1072xx	101xx	1058	10.5	110.0xx		23.4±1.3
3	Control	9	10	1707	151	765	5.4			
	Control + glucose		0	1668	135	615	4.6			
	0.85 units insulin		10	1735	158	752	4.8			
	0.0015% *dl*-thyroxine in diet		18	1153xx	5xx	766	30.5			
	Thyroxine + 0.85 units insulin		10	b 1396xx	b 102xx	764	7.5			
	Thyroxine + 1.70 units insulin		30	1270xx	b 84xx	723	13.3			
4	Control	9	0	1711	182	736	4.0	134.0		
	0.88 units insulin		0	1623	157	700	4.7	131.0		
	0.0015% *dl*-thyroxine in diet		5	1415xx	19xx	856	65.7	130.5xx		
	Thyroxine + 0.88 units insulin		10	1381xx	b 40xx	853	23.4	128.8xx		
	Thyroxine + 1.76 units insulin		5	1422xx	b 80xx	892	8.2	129.1xx		
5	Control	11	0	2213	167	910	5.8			
	0.88 units insulin		5	2156	170	830	4.5			
	0.0015% *dl*-thyroxine→0.003%† in diet		35	1653xx	−31xx	1089	∞			
	Thyroxine + 0.88 units insulin		15	a 1811xx	b 74xx	1113	17.1			
	Thyroxine + 1.76 units insulin		20	1723xx	b 80xx	1051	17.1			
6	Control	11	0	1739	162	794	4.9			
	0.00085% *l*-thyroxine in diet		25	1180xx	83xx	792	9.5			
	Thyroxine + 3.5 units insulin		25	1321xx	a 112xx	813	7.3			

NOTE: NHXBR male chicks were used in all experiments, 10 per replicate, two replicates per treatment.

Replicate values were averaged.

Insulin dosage was increased by 0.1 unit for each 100 g increase in body wt. for the lower level and by 0.2 units for the higher level based on weekly weighings. The dosages listed were the final levels. Glucose was given concurrently with insulin in the amount of 0.34 g/unit insulin. The lower level of glucose was given to the control groups where indicated.

In two preliminary 3-week trials the epiphyseal cartilage plate widths, expressed in mm were as follows: controls 1.08, 0.96; thyroxine-treated 0.77xx, 0.82x.

$P < 0.05$ by analysis of variance (x = difference from control, a = difference from thyroxine).

$P < 0.01$ by analysis of variance (xx = difference from control, b = difference from thyroxine).

ns variation not statistically significant.

*Av. plasma protein-bound iodine values for two pooled replicate samples.

†Thyroxine level was increased after ninth week.

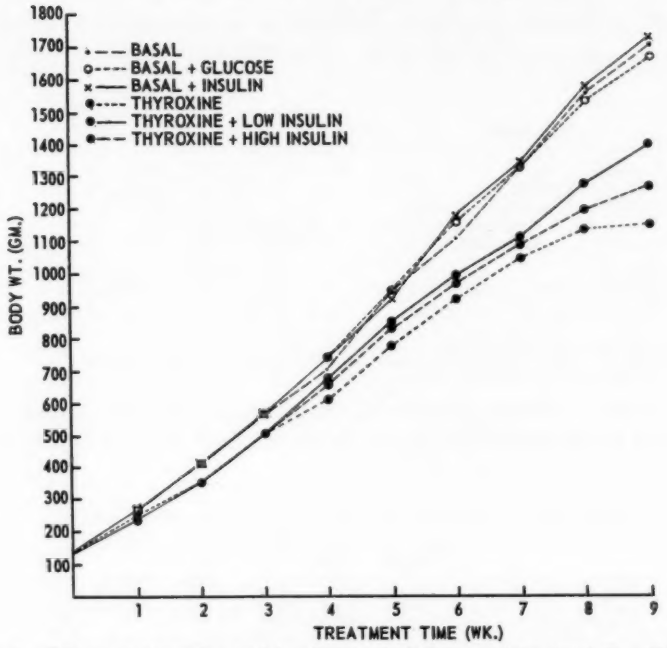

FIG. 1. Effects of thyroxine and insulin on chick body weight (trial 3). The feed consumption data were:

Treatment	Total av. feed consumption (g)	Feed/gain (g/g)
Basal diet	4776	3.05
Basal + glucose	4670	3.06
Basal + insulin	5004	3.14
Thyroxine diet	4732	4.66
Thyroxine + low insulin	4918	3.92
Thyroxine + high insulin	4862	4.31

unit gain of weight which occurred in the thyroxine-treated group tended to be reduced following the concomitant administration of insulin. However, insulin treatment did not affect the elevated plasma protein-bound iodine level which resulted from thyroxine treatment nor did it affect the reduced tibia length which occurred following this treatment. It did not prevent liver glycogen depletion due to excess thyroxine even when the body weight was restored to normal. Neither thyroxine nor insulin administration resulted in any change in the water concentration of the skeletal muscle although the fat concentration was less for chicks receiving these hormones (Table II). Because of the marked resistance of the chick to thyroxine, shown by the lengthy treatment period required to produce a pronounced reduction in rate of body weight gain, and also because of extreme seasonal variations in response, the rat was used in subsequent experiments.

TABLE II

Partial analysis of chick skeletal muscle

Trial No.	Treatment	Av. moisture (%)	Av. protein (N × 6.25) (%)	Av. fat (%)
5	Control	77.1±0.2*		
	0.88 units insulin	76.2±0.2		
	0.0015% *dl*-thyroxine in diet	77.3±0.1		
	Thyroxine + 0.88 units insulin	75.6±0.0		
	Thyroxine + 1.76 units insulin	76.1±0.1		
6	Control	77.1±0.1†	21.8±0.7	3.7±1.0
	0.00085% *l*-thyroxine in diet	78.2±0.6	21.0±0.3	0.6±0.1
	Thyroxine + 3.5 units insulin	77.4±0.2	20.9±0.5	0.8±0.2

*Average values for two replicate groups.
†Average values for four determinations on specimens pooled from all animals in the group. Variation is expressed as standard deviation.

Some of the results of the study of the interrelationship of excess thyroxine and insulin in the metabolism of the rat are shown in Table III. As with the

TABLE III

Insulin mitigation of growth inhibition in the thyroxine-treated rat

Trial No.	Treatment	Length of treatment (wk)	No. and sex of test animals	Mortality (%)	Av. final body wt. (g)	Av. wt. gain for final wk (g)	Av. feed intake for final wk (g)	Av. feed/gain for final wk (g/g)
7	Control	3	10♂	0	133	20	89	6.7
	0.009% *l*-thyroxine in diet		15	0	103xx	10xx	127	12.7
	Thyroxine + 0.5 units insulin		20	13	ns 109xx	ns 13xx	128	9.8
	Thyroxine + 0.75 units insulin		10	20	b 117xx	b 17ns	128	7.5
8	Control	3	8♂	37	105	26		
	1.0 unit insulin		8	12	102	25		
	0.005% *l*-thyroxine in diet		8	37	77xx	10xx		
	Thyroxine + 0.4 units insulin		8	62	b 93x	b 18xx		
	Thyroxine + 0.8 units insulin		8	62	ns 86xx	b 17xx		
	Thyroxine + 1.0 unit insulin		8	62	a 89x	b 19xx		
9	Control	5	12♂	8	150	22		
	0.4 units insulin		12	8	158	30		
	0.004% *l*-thyroxine in diet		12	33	96xx	7xx		
	Thyroxine + 0.2 units insulin		12	33	b 112xx	b 18ns		
	Thyroxine + 0.4 units insulin		12	33	b 130xx	b 19ns		
10	Control	8	15♂	0	236	16	124	7.8
	0.4 units insulin		15	0	225	16	124	7.8
	0.004% *l*-thyroxine in diet		15	26	151xx	7xx	163	23.3
	Thyroxine + 0.2 units insulin		15	66	ns 163xx	ns 6xx	176	29.3
	Thyroxine + 0.4 units insulin		15	73	ns 171xx	b 15ns	172	11.4
11	Control	5	20♂	0	134	24		
	0.01% *l*-thyroxine in diet		15	0	93	9xx		
	Thyroxine + high glucose		10	0	99xx	7xx		
	Thyroxine + 0.32 units insulin		15	40	b 121xx	b 21ns		
	Thyroxine + 0.64 units insulin		15	40	b 116xx	b 17x		

NOTE: Insulin dosage was increased by 0.02 units for each 15-g increase in body wt. for the lower level and by 0.04 units for the higher level based on weekly weighings. The dosages listed were the final levels.
Glucose was given concurrently with insulin in the amount of 0.34 g/unit insulin.
P <0.05 by analysis of variance (x = difference from control, a = difference from thyroxine).
P <0.01 by analysis of variance (xx = difference from control, b = difference from thyroxine).
ns variation not statistically significant.

chick, these data indicate that when thyroxine was given in sufficient quantity over a long enough period of time a persistent, statistically significant decrease (P <0.01 by analysis of variance) in the rate of body weight gain occurred. In all cases the rate of gain was significantly higher than for the thyroxine-

treated group ($P < 0.01$ by analysis of variance) when insulin was administered concurrently. Figure 2 illustrates that these effects occurred throughout the

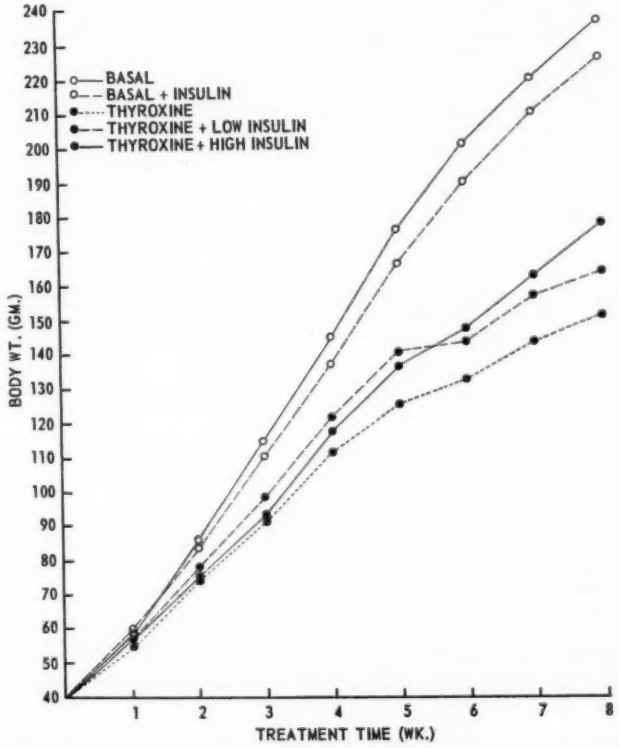

FIG. 2. Effects of thyroxine and insulin on rat body weight (trial 10). The feed consumption data were:

Treatment	Total av. feed consumption (g)	Feed/gain (g/g)
Basal diet	802	4.05
Basal + insulin	783	4.20
Thyroxine diet	981	9.05
Thyroxine + low insulin	1077	8.74
Thyroxine + high insulin	1059	7.92

treatment period. Feed efficiency was reduced as the result of thyroxine administration and this too was largely prevented when insulin was administered concurrently with thyroxine.

The data presented in Tables V and VI illustrate the effects of treatment on skeletal muscle nucleic acids and nuclei concentration. It may be noted

TABLE IV

Partial analysis of rat skeletal muscle

Trial No.	Treatment	Av. moisture (%)	Av. protein (N × 6.25) (%)	Av. fat (%)
9	Control	73.4	18.8±1.0	2.3
	0.4 units insulin	74.0	18.5±0.7	2.7
	0.004% *l*-thyroxine in diet	72.5	19.4±0.4	0.6
	Thyroxine + 0.2 units insulin	72.5	19.8±0.8	0.9
	Thyroxine + 0.4 units insulin	73.0	19.6±0.5	1.0

NOTE: Moisture and fat values were derived from pooled samples comprising all of the leg muscle from each animal.
Variation is expressed as standard deviation.

TABLE V

Effects of thyroxine administration on rate of gain, muscle nucleic acids, nuclei concentration, and blood glucose in the rat

Trial No. 12	Control	0.01% *l*-thyroxine + glucose
Av. final body wt. (g)	178	107
No. of animals	10	8
Av. wt. gain for final wk (g)	20	3
Av. wt. gain for final 3 wk (g)	68	15
Av. ribose of muscle RNA (mg/100 g fresh tissue)	26.1±0.6	32.0±0.3
Av. desoxyribose of muscle DNA (mg/100 g fresh tissue)	8.2±0.5	15.4±0.6
Av. no. of muscle nuclei (10^6/g)	48.2±0.7	97.3±0.4
Av. desoxyribose per nucleus ($\mu g \times 10^{-6}$)	1.7	1.6
Av. muscle nuclear diam. (μ)	12.2±0.1	12.2±0.1
Av. blood glucose (mg%)	101 ±1.2	134 ±8.3

NOTE: A growth inhibitory effect due to thyroxine was evident only during the last 3 weeks of this 6-week trial.
Pooled muscle samples comprising all animals in the group were analyzed in triplicate.
Variation is expressed as standard error of the mean.
The glucose dosage was increased, based on weekly weighings, up to 0.22 g for the final week.

that the reduced body weight which occurs in the thyroxine-treated animal is accompanied by increases in the amount of desoxyribonucleic acid (DNA) and in the number of nuclei per gram of skeletal muscle. The quantity of DNA per nucleus and the diameter of the nucleus are not changed as the result of treatment. The DNA content per nucleus is in good agreement with values reported by Thompson *et al.* (33) and Leuchtenberger *et al.* (18) for various rat tissues. Although the DNA content per unit mass of muscle would appear to be affected inversely as the body weight the DNA content per muscle bundle does not appear to have changed. No decrease occurred in the ribonucleic acid (RNA) content per gram of muscle due to thyroxine treatment. The administration of insulin to the thyroxine-treated animal in sufficient amount to permit a more normal rate of body weight gain also resulted in an approximately normal concentration of muscle DNA. The muscle bundle weight per 100 g body weight was not altered following any of the treatments (Table VI). The constancy of this ratio despite marked changes in body weight shows that this representative muscle mass reflects the weight changes in the whole animal. It is apparent that the changes in muscle weight were

TABLE VI

Effects of insulin administration on rate of gain and skeletal muscle DNA content of thyroxine-treated rats

Trial No. 8	Control	Control + 1.0 unit insulin	0.005% *l*-thyroxine in diet	Thyroxine + 0.4 units insulin	Thyroxine + 0.8 units insulin	Thyroxine + 1.0 unit insulin
Av. body wt. (g)	105	102	77	93	86	89
Av. wt. gain for final wk (g)	26	25	10	18	17	19
Av. muscle bundle wt. (g)*	3.9±0.6	4.0±0.3	2.8±0.6	3.3±0.3	3.1±0.3	3.1±0.3
Muscle bundle wt./100 g body wt*	3.7±0.6	3.9±0.5	3.6±1.0	3.6±0.2	3.7±0.3	3.6±0.4
Av. desoxyribose of muscle DNA† (mg/100 g fresh tissue)	10.5±0.3	11.5±0.4	19.1±1.1	13.5±0.5	12.1±0.5	11.5±0.6
Av. desoxyribose of muscle DNA (mg/muscle bundle)	0.41	0.46	0.53	0.45	0.37	0.36
Av. muscle moisture (%)†	74.5±0.5	74.0±0.1	73.1±0.3	73.4±0.4	73.1±0.3	73.7±0.4

NOTE: A growth-inhibitory effect due to thyroxine was evident throughout the 3 weeks of this trial.
The analytical data were derived from the same pooled muscle bundles comprising all animals in the group.
*Variation expressed as standard error of the mean.
†Variation expressed as standard deviation.

not accounted for by changes in water, protein, or fat concentration (Tables IV and VI).

It appears that the changes in body weight and muscle DNA concentration which occur in the animal treated with excess thyroxine do not occur when insulin is given concurrently. The question arises as to whether the same body weight and DNA changes occur in the diabetic state. Since chicks failed to develop diabetes following alloxan treatment, confirming the findings of Sturkie (31), experiments were conducted with alloxan diabetic rats and the results are shown in Tables VII and VIII. It is apparent that the elevated blood sugar level of the diabetic state was accompanied by a reduction in body weight and by increases in the amount of DNA and in the number of nuclei

TABLE VII

Effects of insulin administration on rate of gain, skeletal muscle DNA, and blood glucose of the diabetic and thyroxine-treated diabetic rat

Trial No. 13	Control	Alloxan diabetic*	Alloxan + 2.0 units insulin	0.004%‡ *l*-thyroxine in diet	Alloxan† thyroxine	Alloxan thyroxine + 2.0 units insulin
Mortality (%)	0	0	0	0	12.5	37.5
Av. body wt. (g)	115	75	102	101	66	86
Av. wt. gain for final wk (g)	19	5	26	12	−6	8
Av. blood glucose (mg%)"	93 ±3.5	407 ±69.3	136 ±28.1	76 ±6.2	274 ±22.9§	129 ±29.3
Av. desoxyribose of muscle DNA" (mg/100 g fresh tissue)	10.3±0.7	16.3±0.3	10.1±0.1	10.6±0.2	18.5±0.9	11.5±0.2
Av. moisture content of leg muscle, %¶	75.4±0.8	75.8±0.3	74.3±0.9	73.8±0.4	73.0±0.6	72.8±0.4

NOTE: Two weeks after alloxan injection the diabetic animals were allotted to the above treatment groups for 1 week.
Each treatment group consisted of eight rats, four of each sex.
Pooled muscle samples comprising all animals in the group were analyzed for DNA and moisture content.
*Two animals in this group were no longer diabetic at the end of the trial.
†One animal in this group was no longer diabetic at the end of the trial.
‡This level of thyroxine was not effective in the time interval involved.
§Variation from alloxan diabetic group was statistically significant at $P < 0.01$ by analysis of variance.
"Variation expressed as standard error of the mean.
¶Variation expressed as standard deviation.

TABLE VIII

Effects of insulin administration on rate of gain, muscle nucleic acids, and nuclei concentration in the diabetic and thyroxine-treated diabetic rat

Trial No. 14	Control	Alloxan diabetic	Alloxan + 1.5 units insulin	0.0075%* *l*-thyroxine in diet	Alloxan thyroxine	Alloxan thyroxine + 1.5 units insulin	Alloxan thyroxine + 2.25 units insulin
Mortality (%)	0	18	18	0	17	36	25
Av. body wt. (g)	141	57	83	128	63	74	72
Av. wt. gain for final wk (g)	24	1	10	17	7	8	12
Av. blood glucose (mg%)	119 ±1.8	527 ±10.6	360 ±21.9	84 ±0.4	248 ±2.8†	270 ±7.8	279 ±3.8
Av. ribose of muscle RNA (mg/100 g fresh tissue)	37.2±1.0	38.1±0.9	33.6±1.1	34.4±0.6	46.2±0.6	39.1±0.3	43.3±1.0
Av. desoxyribose of muscle DNA (mg/100 g fresh tissue)	11.6±0.5	30.8±0.9	14.3±0.3	11.2±0.3	25.4±0.4	21.0±0.8	18.7±0.6
Av. no. of nuclei (10^6/g)	63.8±1.65	155.0±2.59	75.8±1.43	78.6±2.08	150.8±5.70	109.8±5.50	116.8±4.69
Av. desoxyribose/nucleus ($\mu g \times 10^{-6}$)	1.8	2.0	1.9	1.4	1.7	2.0	1.6
Av. nuclear diam. (μ)	11.4±0.1	11.4±0.1	11.3±0.1		11.5± .1	12.3± .1	11.4± .1

NOTE: Two weeks after alloxan injection the diabetic animals were allotted to the above treatment groups for 2 weeks.
Each treatment group consisted of 10 rats, 5 of each sex.
Pooled samples comprising all animals in the group were used for all muscle analyses.
Variation is expressed as standard error of the mean.
*This level of thyroxine was not effective in the time interval involved.
†Variation from alloxan diabetic group was statistically significant at $P < 0.01$ by analysis of variance.

per gram of skeletal muscle. The quantity of DNA per nucleus and the diameter of the nucleus were not changed. The RNA concentration of the muscle was not reduced. The administration of insulin in sufficient amount to restore the blood glucose level to normal resulted in a return of body weight and muscle DNA concentration towards normal. The superimposition of thyroxine in the diabetic rat resulted in no further change in body weight or muscle DNA concentration although blood sugar levels were significantly reduced ($P < 0.01$ by analysis of variance). Insulin treatment again resulted in a return of body weight and muscle DNA concentration towards normal although these differences were not as pronounced for the same amount of insulin as in the absence of exogenous thyroxine.

Discussion

The reported findings support the idea that thyroxine and insulin are related with respect to their metabolic functions. This relationship is not direct since the administration of insulin did not affect the increased plasma protein-bound iodine levels resulting from the administration of excessive thyroxine. It has been shown that the administration of insulin prevents retardation of body weight gain in the alloxan diabetic rat and in the rat and chick treated with excessive thyroxine. Treatment with insulin prevents the reduction in muscle mass which results from thyroxine treatment. The administration of insulin also prevents the increases in the amount of DNA and in the number of nuclei per unit mass of skeletal muscle which accompany reduction in body weight gain in both of these conditions.

It would appear that DNA content is not a reflection of muscle mass since (*a*) the DNA content per muscle bundle is unchanged as the result of any of the treatments, (*b*) muscle bundle weight changed proportionately with body weight as the result of the various treatments, and (*c*) changes in muscle mass were not accounted for by changes in its water, protein, or fat concentration.

The decreased rate of body weight gain and reduced muscle mass which resulted from the administration of excessive thyroxine were accompanied by a decreased feed efficiency. The administration of insulin to the thyroxine-treated animal reduced this wastage of food energy. Obviously, some mechanism must be sought for the action of insulin other than that on the intake of energy sources.

If one may be permitted to speculate, it appears that the two hormones may be involved in control of rate of oxidative phosphorylation. It is possible that in the normal animal the level of thyroid hormone activity must be balanced by a certain amount of insulin since increase in thyroid secretion results in an increase in insulin requirement. An excessive amount of thyroxine produces toxic effects, in part, because the pancreas cannot produce sufficient insulin to balance the thyroxine level. Exogenous insulin can satisfy this increased demand and thereby largely prevent the toxic effects of excessive thyroxine. Insulin would appear to exert its effect on energy uptake, possibly through an increased supply of phosphorylated intermediates.

Evidence for a divergent channelling of glucose due to thyroxine and insulin action is shown by the fact that both hormones exert a hypoglycemic effect both in the diabetic and in the normal animal. The basic natures of these mechanisms for glucose utilization remain obscure and must await elucidation of the respective roles of the two hormones. However, any theory which is advanced to explain their respective roles will have to take their interrelationship into consideration.

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SOME PROPERTIES OF THROMBIN PREPARATIONS[1]

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Abstract

Bio-resin thrombin preparations were found to contain three weak precipitinogens. The clotting activity was not demonstrably associated with the precipitinogenic systems. Further, work was done on methods for the purification of citrate resin thrombin, and its clotting activity is also not associated with a precipitinogenic system. The N-terminal amino acid of both bio-resin thrombin and citrate resin thrombin was found to be glutamic acid. The two preparations were found to be homogeneous upon ultracentrifugal examination and could not be differentiated on the basis of sedimentation constants. Since "citrate" activation and "bio" activation produce eventually similar thrombin material, the autocatalytic activation of prothrombin in 25% sodium citrate solution can be used as an ideal model of prothrombin activation. The prothrombin first dissociates to form a derivative that does not form thrombin in the two-stage analytical reagents. Then a second alteration occurs in which the derivative again may form thrombin in the two-stage analytical reagents. Then thrombin activity appears as esterase activity, then as clotting activity. Later the clotting activity may be lost and finally also the esterase activity. The original prothrombin is a precipitinogen while the active thrombin is not.

Introduction

Purified prothrombin can be placed in 25% sodium citrate solution and converts itself to thrombin by autocatalysis (1, 2). This discovery is consistent with the view that prothrombin itself contains all that is required to form thrombin and that the active enzyme, thrombin, is an activator of prothrombin. This autocatalytic process can readily be inhibited at an intermediate stage with certain organic compounds or with material in plasma or serum that is adsorbable on barium sulphate (1–4). In this way it is possible to stop the activation so that no appreciable quantity of thrombin accumulates. Some prothrombin preparations that are polydisperse upon ultracentrifugal analysis have reached only an intermediate stage with sodium citrate activation (5).

The fact that only thrombin is necessary for the activation of prothrombin has implications for our understanding of all prothrombin activations. The number of clotting factors has rapidly increased so that we might wonder about the significance of any one of them. Some are said to be "essential" for prothrombin activation while the terms "activator" or "accelerator" are frequently used in characterizing a favorite clotting factor. In a recent review (6) the following remark appears: "Activation by biological factors and calcium ions does not seem to require thrombin as a catalyst." Evidently the vast

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literature tends to obscure the fact that thrombin itself is of prime importance in any prothrombin activation.

The transformation of prothrombin to thrombin is a degradation process already studied to a certain extent and now accessible for a more detailed description in terms of chemistry, because methods have been devised for the purification of prothrombin and thrombin (7–13). Technics developed in protein chemistry are also ready to be applied. We regard it as quite significant that no one has found and characterized as an enzyme any substance amongst the blood coagulation factors. Brain thromboplastin, long regarded by some authors as an enzyme, has now been prepared free of protein (14). Thrombin, however, is present in all activations where prothrombin is converted to thrombin, and it is an enzyme. On the evidence from studies with purified prothrombin a basic generalization has been stated, namely, that thrombin is more effective for furthering the conversion of prothrombin to thrombin in the presence of certain procoagulants than by itself, and less effective when anticoagulants are interfering (15).

In this paper we describe briefly our inquiry into the nature of thrombin preparations obtained by methods recently described (13). In one instance the enzyme activity was developed by autocatalysis in 25% sodium citrate solution to get citrate thrombin. It was also developed in the presence of calcium ions, Ac-globulin, and lung thromboplastin. This latter activation gives biothrombin with a sedimentation constant approximately equal to prothrombin itself (16, 17), but upon passing through an Amberlite IRC-50 (XE-64) column it separates and one of the components has thrombin activity. This is bio-resin thrombin, and in the work described below it was indistinguishable from citrate resin thrombin on the basis of sedimentation in an ultracentrifuge, N-terminal amino acid analysis, and immunochemical procedures. Thus, in either activation with sodium citrate or activation in the presence of procoagulants from plasma and tissues, approximately the same work is accomplished, and according to our view thrombin is the activator.

Materials and Methods

Prothrombin was separated from bovine plasma (11) and from this the following were prepared as described previously: *citrate thrombin* (17), *biothrombin* (17), *bio-resin thrombin* (13), and *citrate resin thrombin* (13). *Prothrombin activity* was assayed by the two-stage procedure as modified by Ware and Seegers (18). *Thrombin activity* was determined by clotting of fibrinogen (19) and by hydrolysis of *p*-toluene sulphonyl-L-arginine methyl ester (20).

For immunochemistry work previous description has been given of the technic involved in the micro-precipitin ring test, precipitin reaction, and immunization procedure (21); for double diffusion in agar a modification of Ouchterlony's method was used (22). The gel was a 2% heart infusion agar (Difco) – 0.01% merthiolate mixture.

For the ultracentrifuge observations a Spinco Model E Analytical Centrifuge was used. In the double-sector cell the solvent, 0.1 *M* KCl, was placed on one

side and the thrombin in 0.1 *M* KCl on the other. All runs were made at 59,780 r.p.m. and at 20° C. The reversible cooling on acceleration observed by Waugh and Yphantis (23), and others was taken into account by starting the acceleration at 20.9° C. The photographic plates were read by a projection comparator with a precision corresponding to a 5-μ movement of the boundary in the centrifuge cell.

To determine the N-terminal amino acids we used the methods of Edman (24), Sjöquist (25), and Edman and Sjöquist (26) as employed by Blombäck and Yamashina (27). For chromatography of the phenylthiohydantoins derivatives (PTH) we used the following: solvent A, *o*-xylene; solvent B, 40 ml of *n*-heptane, 40 ml of *n*-butanol and 20 ml of *n*-heptane and 40 ml of formic acid; solvent C, 40 ml of *n*-heptane, 40 ml of butanol, and 20 ml of formic acid 90%. The lower phase was used for chamber equilibration while the upper phase was put into the trough; solvent D, one volume of *n*-butyl acetate and one volume of *o*-xylene saturated with water and formamide. To 30 volumes of this mixture was added one volume of propionic acid; solvent E, *n*-butyl acetate was saturated with water and 3% propionic acid was added and the mixture was saturated with formamide; solvent F, 1 volume of *n*-heptane, 2 volumes of 75% formic acid, and 2 volumes of ethylene chloride; solvent G, 2 volumes of 75% formic acid, 1 volume of ethylene chloride, and 1 volume of *n*-heptane. For both solvent F and G the lower phase was used for chamber equilibration while the upper phase was put into the trough.

Immunochemical Studies

A single rabbit was injected subcutaneously with a total of 267,300 units (about 67 mg) of bio-resin thrombin. The inoculation schedule and thrombin preparations are described in Table I. The total unitage injected represents an

TABLE I

Inoculation schedule for bio-resin thrombin*

Day	Thrombin preparation	Volume, ml	Units
1	580618†	3.0	99,000
8	580618	2.8	92,400
18	580708‡	3.0	75,900

*Incorporated in a Freund adjuvant emulsion and injected subcutaneously into a 3.6-kg rabbit. Exsanguination was performed on day 28.
†42,000 units/mg tyrosine.
‡36,600 units/mg tyrosine.

enormous quantity when referred to other injection experiments involving thrombin. The immunization technique is known to yield high-titered antisera even with relatively weak antigens. Nonetheless, a low-titered antiserum was recovered from the rabbit.

The mixture of antiserum and bio-resin thrombin produced a faint precipitate. It was detectable in both gel diffusion and micro-precipitin ring tests. In the double diffusion technic a single, faint band was visualized (Fig. 1).

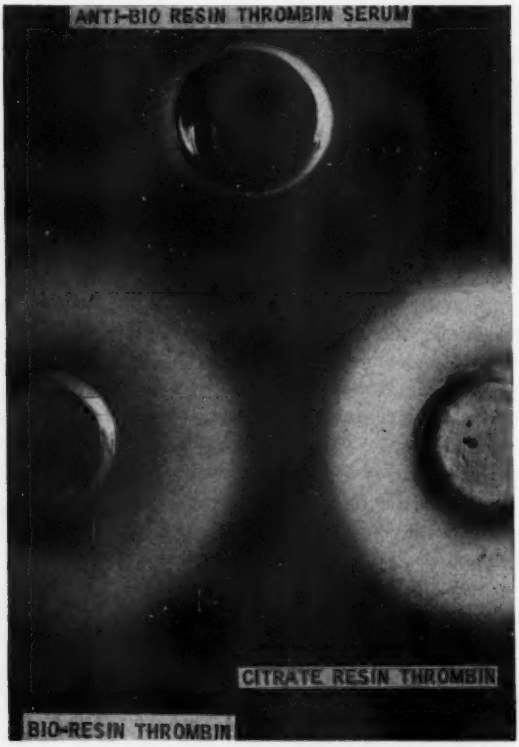


FIG. 1. Agar diffusion plate with (1) anti-bio-resin serum, (2) bio-resin thrombin (80,000 units/ml), and (3) citrate resin thrombin (80,000 units/ml). Photograph was taken on 22nd day. The opaque areas around the thrombin wells are typical of these thrombin preparations.

This was the case even when a concentration ranging up to 80,000 units/ml was employed. In order to determine if clotting activity was bound to the precipitate, resort was made to activity measurements of supernatant solutions from precipitin tests. These data are recorded in Table II. Activity was removed to the same extent by both normal and immune sera. In both instances the natural antithrombin of the plasma accounts for the thrombin activity neutralized. No basis was found for association of the clotting and precipitinogenic activities of bio-resin thrombin preparations. Similar observations were made on citrate resin thrombin of the kind described below.

We deduce the presence of two additional components in bio-resin thrombin. Highly purified, but still heterogenous preparations of prothrombin and biothrombin were reacted with the antiserum to bio-resin thrombin. Up to three components were seen.

With another approach only one component seemed to be present. Antisera were produced against prothrombin, biothrombin, and plasma. The antisera were matched against bio-resin thrombin (up to 80,000 units/ml) and in each

TABLE II

Thrombin assays on supernatants from the precipitin reaction with bio-resin thrombin and its specific antiserum

Total thrombin units		
	Remaining	
Added	Antiserum	Control serum
2550	1680	1600
1420	710	800
700	270	270
600	280	300
380	28	37
245	1	1
200	0	0
141	0	0
85	0	0

case only a single faint band was seen. A band of identity formed when bio-resin thrombin together with either biothrombin, prothrombin, or whole plasma were reacted with any one of the antisera. By this criterion, the single band formed in the bio-resin thrombin systems is identical with one common to biothrombin, prothrombin, and plasma systems. These observations are interpreted by considering that the precipitinogenic plasma components are carried through the process of purification and are present in their lowest relative concentration in the bio-resin thrombin preparations.

Through application of these immunochemical procedures the following characteristics of bio-resin thrombin are found: (1) bio-resin thrombin preparations served as relatively weak precipitinogens; (2) they possessed clotting activity which was not demonstrably associated with the precipitinogenic systems; (3) they possessed a minimum of three precipitinogenic components, some of which bear relation to components present in biothrombin and prothrombin preparations and whole plasma.

Thrombin Preparation

In the work of Seegers, Levine, and Shepard (13) very little information was available about the purification and properties of citrate resin thrombin. Our experience has been far more extensive. In accordance with previous descriptions we repeatedly succeeded in getting a thrombin fraction of high potency. First the sodium citrate activated prothrombin was fractionated with sodium citrate (17), precipitated with acetone, taken up in buffer, and passed through an IRC-50 (XE-64) resin column, precipitated with ammonium sulphate at 75% of saturation, dissolved in water, and thrice precipitated in 50% cold acetone to remove salts. The precipitated thrombin was then usually dissolved in 0.1 *M* KCl.

The specific activity was determined in terms of units/mg dry weight, units/mg N, and units/mg tyrosine (Table III). In all three ways of measuring, the activity was a little less than found for bio-resin thrombin as previously

TABLE III

Some properties of citrate resin thrombin

Thrombin product No.	Clotting activity		
	Tyrosine units/mg	Nitrogen units/mg	Dry weight units/mg
580311	30,200	31,500	—
580724	34,000	37,000	—
580730	30,000	30,000	—
580805	30,000	36,500	3,650
580821	34,000	32,000	3,800
580611	30,000	—	3,340

reported (13). This difference might be important; however, we are not certain. In some additional comparisons bio-resin thrombin and citrate resin thrombin were prepared by the same person. The citrate resin thrombin was a little less active.

The clotting activity of thrombin can be dissociated from the esterase activity (28, 29). Accordingly we compared the relationship of esterase activity to clotting activity in citrate resin thrombin. The ratio of these activities, as found in five different biothrombin preparations, was arbitrarily designated as unity. By this criterion all citrate resin thrombin preparations had a ratio of one when freshly prepared. At 4° C the clotting activity was stable for more than 6 months; however, at room temperature it began to drop within 2 days. The esterase activity remained at the original level until the sixth day and then began to drop. Thus, the esterase and clotting activity can be dissociated in citrate resin thrombin just as it can in biothrombin or bio-resin thrombin.

Some qualitative or semiquantitative observations were made on the solubility of citrate resin thrombin. A solution containing 25 mg/ml could easily be made with 0.9% NaCl or 0.1 *M* KCl. If the saline solution was diluted about 40–50 times with water, cloudiness was noticed and a small precipitate could be centrifuged down. When taken up in water the thrombin activity of the dissolved precipitate was a little higher than that of the original. We also found that there seems to be a definite low point of solubility around pH 5.2. This was observed in the following experiments: the thrombin was dissolved in citrate–phosphate buffer systems of pH range from 4 to 7. Alcohol was added to develop turbidity and this was most easily achieved at pH 5.2.

N-Terminal Amino Acid Studies

Bio-resin thrombin was coupled, cycled, and hydrolyzed as in the PTH method. The chromatography with solvent E led us to the conclusion that the N-terminal amino acid is glutamic acid, the R_f value being 0.71. With solvents A, B, C, F, and G the decision between glutamic acid and the possibility of threonine could be made in favor of glutamic acid, but with solvent A the two amino acids were indistinguishable. Some of the chromatographed hydantoin was extracted from the filter paper with alcohol and the absorption

maximum in the ultraviolet (Beckman Model DU) was found to be 271 mμ. This corresponds to our observations with the hydantoin of glutamic acid. On a recording instrument the value 270 mμ was found. The chromatographed material was easily soluble in chloroform.

Citrate resin thrombin was studied in the same way. Only one N-terminal amino acid was found; namely, glutamic acid. We thus find that this property of thrombin is the same whether the prothrombin activation was in 25% sodium citrate solution or by means of calcium ions, lung thromboplastin, and Ac-globulin. We further determined that the quantity of this amino acid is about the same in the two. In each instance the chromatographed hydantoin was extracted with alcohol and the concentration determined by ultraviolet absorption at 271 mμ. The concentration of glutamic acid in bio-resin thrombin was 5.25% (free amino acid basis) and in citrate resin thrombin it was 5.3%. These results are not corrected for loss in yield due to any failure of coupling with phenylisothiocyanate or destruction resulting from cyclization. Later we hope to determine more accurately the total quantity of N-terminal glutamic acid. From our work thus far we know the minimum concentration and that it is the same in the two thrombin preparations. Both were treated in the same way. Perhaps thrombin has most of its glutamic acid in the form of N-terminal residues.

Ultracentrifuge Studies

All the thrombin preparations examined were found to be homogeneous, and from the performed determinations could not be differentiated on the basis of sedimentation constants (Table IV).

TABLE IV

Sedimentation data for thrombin preparations

Thrombin	Preparation No.	Concentration, per cent (w/v)	S_{20}
Bio-resin	580626	0.85	3.5
	580101	0.55	3.9
	580814	0.2	3.3
Citrate resin	580307	0.9	3.3
	580611(2)	0.3	3.8
		0.09	3.6
Bio-resin*	580703	0.9	3.8
	580718	0.25	3.4
Bio-resin†	580611(1)	0.9	‡
	580611(1)	0.3	3.5
	580611(1)	0.09	3.4

NOTE: S_{20} = Sedimentation constant in units of 10^{-13} sec at 20° C and at the concentrations indicated.
*Placed in 25% sodium citrate solution then freed of citrate and taken up in 0.1 *M* KCl.
†Placed in 25% sodium citrate solution and again passed through IRC-50 (XE-64) column.
‡Qualitative information only — single peak.

In previous work (13) it was found that bio-resin thrombin could be dissolved in 25% sodium citrate solution, and that some tyrosine-containing material

would become soluble in trichloracetic acid. We wanted to see whether we could get a smaller particle to have the activity and accordingly dissolved bio-resin thrombin in 25% sodium citrate. After 15 minutes the thrombin was precipitated by bringing the salt concentration to 36%. To remove salts the precipitate was dissolved in water and reprecipitated with 50% (v/v) cold acetone. The average activity of three preparations was 31,300 units/mg tyrosine. This did not significantly change the particle size (Table IV). Then we thought that a hypothetical fragment might now be removed by means of the resin column. Accordingly two preparations of bio-resin thrombin were treated with sodium citrate as described above. The activity was twice precipitated with cold acetone to remove salts. Then the preparations were taken up in buffer and again put through the IRC-50 (XE-64) resin column, and precipitated in the usual way. The two specific activities were respectively 26,200 and 34,600 units/mg tyrosine. Again, within the limitations of our observations, the particle size was not altered (Table IV).

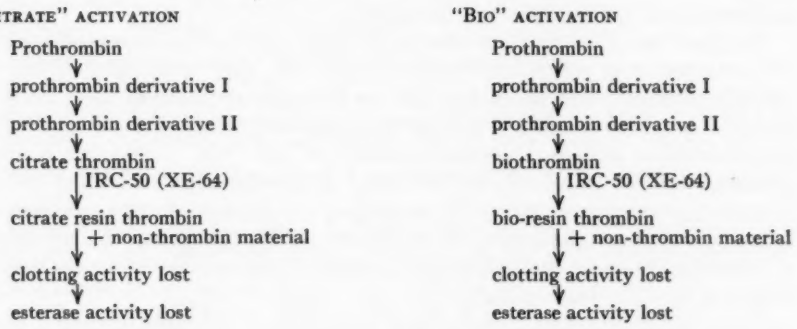
Discussion

The bio-resin thrombin preparations studied contained at least three precipitinogens but these were very weak, and the clotting activity which was very powerful was not associated with any of the precipitinogens. The thrombin molecule, as represented in these preparations, is not a precipitinogen even though it is a protein. The method for detecting the precipitinogens is very sensitive and we infer that the bio-resin thrombin preparation is one of high quality from which one or two more simple purification steps might take away the small amount of remaining non-thrombin protein. By the criterion of ultracentrifugation and N-terminal amino acid analysis homogeneity is indicated. There was only one N-terminal end group and only one symmetrical peak in the ultracentrifuge pattern.

Bio-resin thrombin was not distinguishable from citrate resin thrombin on the basis of ultracentrifugation and N-terminal amino acid analysis. Neither of the two has precipitinogenic properties. Possibly the clotting activity per milligram dry weight was lower for citrate resin thrombin. The uncertainties with respect to this last-mentioned fact are considerable and in view of the end group and ultracentrifuge analysis we regard bio-resin thrombin as being similar to citrate resin thrombin.

The two thrombin products are the highest stage of enzyme development in a series of events related to prothrombin activation. The next known change is the loss of clotting activity and eventual loss of esterase activity. We can compare the two ways in which the thrombin was obtained. For this the "citrate" activation can serve as a model. It is unencumbered by clotting factors which tend to distract attention from the bare essentials of prothrombin activation. Prothrombin activation by autocatalysis in sodium citrate solution breaks the substructure of prothrombin, so that the fractional unit containing the thrombin activity is separated in about the same way as happens in the presence of calcium ions, Ac-globulin, and lung thromboplastin. How-

ever, in the latter activation the subunits of biothrombin reassociate and the particle size is about the same as in prothrombin (16, 17). The separation of the dissociated prothrombin (biothrombin) occurs in passing through the IRC-50 (XE-64) resin column giving thrombin (bio-resin thrombin) free of the cleavage particles. Most likely the first dissociation represents formation of intermediates prior to the development of clotting power. When prothrombin activates itself in sodium citrate solution dissociation also occurs before the thrombin activity appears. Thus, in "citrate" activation and in "bio" activation the large unit is first altered and thereafter thrombin activity develops. Briefly stated the activation of prothrombin goes through a cycle, and the thrombin activity is associated with the same kind of structure whichever way the activation is achieved, and requires no material substance outside of prothrombin itself. An outline of this idea follows:



Firstly, the isolation of prothrombin in purified form established the view that it is a single molecular entity and not a complex in the sense of complement. This is the base for our orientation. The work in this paper makes it possible to see that the thrombin from "citrate" activation is like the thrombin from "bio" activation. In 25% sodium citrate activation is by autocatalysis (1, 2). Early in the activation the electrophoretic mobility decreases (2), the molecule dissociates (30), prothrombin derivative I forms and is insensitive to the procoagulants like calcium ions, Ac-globulin, and thromboplastin. This derivative has accelerator properties (31) in certain prothrombin activations, and changes to prothrombin derivative II and then is again sensitive to calcium ions, Ac-globulin, and thromboplastin (3). Following this, thrombin activity appears. The actual development of thrombin activity can be prevented with 3,4,4'-triaminodiphenyl sulphone (1), soybean trypsin inhibitor (32), or plasma proteins (4). After full development of the clotting activity it may disappear. After that the esterase activity may also be lost. The work described in this paper constitutes evidence that citrate resin thrombin is similar to bio-resin thrombin.

In the "bio" activation the same pattern displayed in "citrate" activation is also seen. However, events are so rapid that special experiments are needed

to see the corresponding details. Again the starting material is prothrombin. It can form a derivative in the presence of thromboplastin and calcium ions (10) or in the presence of platelet factor 3 and calcium ions (33). This is prothrombin derivative I of the above diagram, and it has accelerator qualities in prothrombin activation (34). It is insensitive (not converted to thrombin) to calcium ions plus Ac-globulin plus thromboplastin. It can be converted to thrombin in 25% sodium citrate solution (34). It can change to prothrombin derivative II spontaneously (35) or in the presence of mitochondria[2] (36). Then it may become biothrombin. This has a particle size or degree of aggregation somewhat like prothrombin (16, 17), but differs from prothrombin in that it separates on an IRC-50 (XE-64) column whereas prothrombin does not (12, 13). When the separation is made the non-thrombin portion contains the original precipitinogenic qualities of prothrombin, and the thrombin portion is similar to citrate resin thrombin in that the N-terminal amino acid, and sedimentation constant are the same.

The fact that bioactivation usually goes so rapidly that intermediates are not detected does not prevent the inference that they are there. With the several different ways to deploy and to have the derivatives form there is provided a means to see the details and construct the general pattern. The correspondence between "citrate" activation and "bio" activation is comprehensive. The autocatalytic activation of prothrombin in sodium citrate solution is activation by thrombin, and just as this activation can be accelerated or inhibited by simple organic compounds or plasma proteins so the activation of prothrombin in our physiology is by autocatalysis and can be accelerated or inhibited by "clotting factors".

This way of studying prothrombin activation is uniquely different from previous approaches. It begins centrally, with prothrombin, and works outward to include other substances. Previous approaches *assumed* prothrombin to be a substance or substances and began peripherally by placing emphasis on clotting factors. This exploration included complex serum which contains the materials of spent reactions. This approach has the limitation of not revealing what is inherently concerned with prothrombin and what is primarily concerned with procoagulants or anticoagulants and eventually also with prothrombin. Prothrombin itself is a wonderful construct, and capable of important alterations not imagined before. As this approach to the study of prothrombin is expanded to include observations of procoagulants and anticoagulants we may find that some of the postulated substances are actually manifestations of some property of prothrombin itself.

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[2]There is the possibility that the mitochondria regenerate the original prothrombin structure, but as yet this cannot be decided either from our work or from the work of Lasch and Roka (37).

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EFFECT OF X IRRADIATION ON THE METABOLISM OF C^{14}-GLUCOSE AND C^{14}-FRUCTOSE IN RAT LIVER SLICES[1]

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Abstract

The utilization of C^{14}-glucose and C^{14}-fructose in liver slices from normal rats and from rats exposed to 1000 r of whole-body X irradiation was studied. Liver slices prepared from normal rats were incubated with C^{14}-glucose or C^{14}-fructose in equivalent amounts and the incorporation of C^{14} into carbon dioxide, glycogen, and an ethanolic extract was determined. After the rats had been fasted 4 or 24 hours the amount of C^{14} incorporated into glycogen and carbon dioxide from C^{14}-fructose was greater than that incorporated from C^{14}-glucose but the total radioactivity in the ethanolic extracts was approximately the same for both hexoses. When liver slices prepared from normal and X-irradiated rats were incubated with C^{14}-glucose or C^{14}-fructose 4 or 24 hours after irradiation, the samples obtained from irradiated rats incorporated a greater amount of C^{14} into carbon dioxide, glycogen, and the ethanolic extract, with the exception of the 24-hour samples incubated in the presence of labelled glucose. In the latter instance incorporation into carbon dioxide fell below the normal value. The total C^{14} recovery from the three fractions was always higher in the X-irradiated samples than in the corresponding control samples. Further examination of the ethanolic extracts (amino acids, lactic acid, and origin area material) separated by paper partition chromatography revealed additional differences between the samples of liver from normal and X-irradiated rats in their ability to incorporate C^{14} from either labelled hexose. These differences were apparent in samples incubated either 4 or 24 hours after X irradiation of the animals.

Introduction

It has been demonstrated by various workers that carbohydrate metabolism in the irradiated animal is disturbed. Enormous doses of X rays (110,000 r, survival 3½ hours) clear the liver of its glycogen reserves (1). However, a moderate dose of X rays (300 r) in rats fasted before and after irradiation brought about a considerable and steady increase in the glycogen content of the liver during the hours following irradiation (2, 3, 4). Lourau-Pitres (4) also showed that during the first 2 hours after a dose of 2000 r in mice, the liver takes up three times as much C^{14} in glycogen as the controls after a subcutaneous injection of labelled glucose. Recently Nims and Sutton (5) and Kay and Entenman (6) reported that liver glycogen was increased in the fasted rat after whole-body irradiation. Morehouse and Searcy (7) were able to demonstrate that C^{14} from C^{14}-acetate is more rapidly incorporated into liver glycogen by the fasted irradiated rat than by the fasted non-irradiated control.

Tracer experiments using C^{14}-glucose and C^{14}-fructose provide good evidence that the metabolism of glucose differs from that of fructose in the liver (8, 9) and further evidence is provided by Hill *et al.* (10) that X irradiation produces different effects on glucose and fructose metabolism in animals. This study was carried out to investigate further the effect of X irradiation on the metabolism of C^{14}-glucose and C^{14}-fructose.

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Experimental

Irradiation of Animals

Rats maintained on Purina fox chow and weighing 200–300 g were exposed to 1000 r of X rays under the following conditions: 220 kv; 15 ma; filters 0.5 mm Cu and 1.0 mm Al. The dose rate was 20 r/minute. Food was withdrawn from both the non-irradiated controls and the irradiated rats just before the irradiation period.

Incubation Procedures

The rats were killed by a blow on the head, the livers removed immediately, chilled, and sliced with a Stadie–Riggs slicer. The livers from three animals of the same group were sliced, pooled, and 500 mg of tissue was placed in the outer compartment of a 50-ml incubation flask which contained either C^{14}-glucose or C^{14}-fructose in 5.0 ml of Krebs–Henseleit bicarbonate buffer (11) (pH 7.3–7.4). The uniformly labelled hexoses were prepared by the authors according to the method of Vittorio (12). Incubations and analyses were carried out in triplicate, and each result represents the average value obtained with the livers of three rats. The flasks were gassed with a mixture of 95% O_2 and 5% CO_2. They were then capped with a self-sealing rubber stopper and placed in a Dubnoff metabolic shaker (37.5° C) for 3 hours. At the end of the incubation period approximately 0.5 ml of 10% KOH was injected into the center well of the flask by means of a long needle attached to a hypodermic syringe. In a similar manner 1 *N* HCl was added to the incubation medium (pH 3) to inactivate the tissue.

At least 30 minutes were allowed for CO_2 absorption. The contents of the center well were then transferred to a round-bottom flask and the CO_2 was released *in vacuo* by the addition of acid and trapped in dilute (0.05 *N*) NaOH. The CO_2 was precipitated with $BaCl_2$ as $BaCO_3$ and the total amount of CO_2 present was determined by titrating the excess NaOH with HCl. The $BaCO_3$ was washed with distilled water and plated as a slurry on weighed aluminum trays. The samples which were prepared in triplicate were dried under infrared lamps and reweighed. They were then counted in a methane gas flow counter and the total activity of the respired carbon was calculated.

The liver slices were extracted with hot 80% ethanol so that two fractions, (*a*) the ethanolic extract and (*b*) the liver residue, were obtained.

The Ethanolic Extract

This fraction was chromatographed one-dimensionally in butanol–ethanol–water (52.5-32.0-15.5) solvent and two-dimensionally in phenol–water and butanol – acetic acid – water (9-1-4) solvents. Three chromatograms were run in each solvent. The first was tested with aniline phthalate and orcinol sprays to identify the sugars present. The second was sprayed with ninhydrin to help locate and identify the amino acids present (13) and to determine if any of the sugar spots were contaminated with radioactive amino acids. The third was radioautographed to locate the radioactive areas. The radioactive compounds studied were eluted, plated, and their activities determined as

described elsewhere (14). Both sets of chromatograms were used to obtain the final results. All amino acids containing radioactivity were pooled since individual elutions were not feasible. However, in all cases the same amino acids were included in each group.

The Liver Residue

The glycogen was extracted from the liver slices with hot 30% KOH, precipitated with 95% ethanol, and further purified. The total and specific activity of the glycogen were determined by dissolving it in a known volume of distilled water and plating aliquots on aluminum planchets. These were counted in a methane gas flow windowless counter.

Results

Liver slices were prepared, as described earlier, from normal rats and from rats exposed to 1000 r of whole body X irradiation. Samples were taken 4 and 24 hours after X irradiation. They were incubated with either C^{14}-glucose or C^{14}-fructose and the activity incorporated into carbon dioxide, glycogen, and the ethanolic extract was determined.

Table I compares the incorporation of C^{14} into carbon dioxide, glycogen, and the ethanolic extract when equal amounts of C^{14}-glucose or C^{14}-fructose

TABLE I

The incorporation of C^{14} from equivalent amounts of C^{14}-glucose and C^{14}-fructose into CO_2, glycogen, and the ethanolic extract by liver slices from fasted rats

Fasted, hr	CO_2, %*	Glycogen, %*	Ethanolic extract, %*
4	222	225	98
24	289	161	110

*The results obtained with C^{14}-glucose have been arbitrarily assigned a value of 100% and the results obtained with C^{14}-fructose have been expressed as a per cent of the glucose value.

were incubated with liver slices from fasted rats. When the rats were fasted for 4 or 24 hours the amount of C^{14} incorporated into glycogen and carbon dioxide from C^{14}-fructose was greater than that incorporated from C^{14}-glucose but the total radioactivity in the ethanolic extracts was approximately the same for both hexoses.

Table II shows the incorporation of C^{14} from C^{14}-glucose and C^{14}-fructose into carbon dioxide, glycogen, and the ethanolic extract by liver slices from non-irradiated and irradiated rats. In this table the values obtained with liver slices for the controls (non-irradiated) have been assigned a value of 100% and values for the X-irradiated samples have been expressed as a percentage of the controls. Four hours after X irradiation the incorporation of C^{14} from both labelled hexoses into the respired carbon dioxide was higher than in the non-irradiated controls, but fructose showed a greater percentage increase than did glucose. Twenty-four hours after X irradiation the samples incubated with C^{14}-fructose still respired more C^{14} than the controls but the

TABLE II

The incorporation of C^{14} from C^{14}-glucose and C^{14}-fructose into CO_2, glycogen, and the ethanolic extract by liver slices from non-irradiated and irradiated rats

Incubated with:	Time after irrad., hr	CO_2, %*	Glycogen, %*	Ethanolic extract, %*
C^{14}-Glucose	4	107	300	102
C^{14}-Glucose	24	90	250	131
C^{14}-Fructose	4	128	160	117
C^{14}-Fructose	24	110	133	120

*The values obtained for the controls (non-irradiated) have been arbitrarily assigned a value of 100% and values for the irradiated samples have been expressed as a per cent of the controls.

value approached that of the controls. For the same time period the X-irradiated samples incubated with C^{14}-glucose incorporated less C^{14} into respired carbon dioxide than the controls and C^{14} incorporation by the former appeared to be decreasing at about the same rate as the X-irradiated sample incubated with C^{14}-fructose. Initially, under the conditions of these experiments X irradiation appeared to stimulate the amount of C^{14}-glucose and C^{14}-fructose carbon respired and the stimulation of fructose was greater. At both 4 and 24 hours the liver slices from the X-irradiated rats incubated with either C^{14}-glucose or C^{14}-fructose contained more C^{14} in glycogen than the non-irradiated controls. The irradiated samples incubated with C^{14}-glucose showed a greater percentage increase than the irradiated samples incubated with C^{14}-fructose. This increase in glycogen C^{14} for both irradiated samples incubated with the hexoses appeared to be decreasing with time and the rate of decrease seemed to be about the same for both. Four hours after X irradiation the C^{14} in the ethanolic extract from the irradiated samples incubated with C^{14}-glucose were about the same as the corresponding controls. The X-irradiated samples incubated with C^{14}-fructose showed an increase over the controls. Twenty-four hours after X irradiation both samples incubated with either C^{14}-glucose or C^{14}-fructose incorporated more C^{14} into the ethanolic extract than did the non-irradiated controls.

Table III shows that at both 4 and 24 hours the total activity recovered from the rat liver slices in CO_2, glycogen, and the ethanolic extract, expressed

TABLE III

Incorporation of C^{14}-glucose and C^{14}-fructose by rat liver slices

Substrate	Treatment	Time after irrad., hr	Total* % recovery
C^{14}-Glucose	Non-irrad.	4	75
C^{14}-Glucose	Irrad.	4	77
C^{14}-Glucose	Non-irrad.	24	67
C^{14}-Glucose	Irrad.	24	87
C^{14}-Fructose	Non-irrad.	4	74
C^{14}-Fructose	Irrad.	4	87
C^{14}-Fructose	Non-irrad.	24	74
C^{14}-Fructose	Irrad.	24	89

*This represents the total C^{14} recovered in CO_2, glycogen, and the ethanolic extract expressed as a per cent of the total C^{14} fed.

as a percentage of the activity administered, was higher in the X-irradiated samples.

The ethanolic extracts were chromatographed and radioautographed as already described. The compounds studied were eluted and their activities determined. The results obtained are shown in Table IV. The amount of

TABLE IV

The incorporation of C^{14} from C^{14}-glucose and C^{14}-fructose into constituents of the ethanolic extract by liver slices from non-irradiated and irradiated rats

Incubated with:	Time after irrad., hr	Origin area, %*	Lactic acid, %*	Amino acids, %*	Glucose, %*	Fructose, %*
C^{14}-Glucose	4	37	194	49	104	
C^{14}-Glucose	24	100	14	96	97	
C^{14}-Fructose	4	99	150	86	75	132
C^{14}-Fructose	24	391	155	202	118	134

*The values obtained for the controls (non-irradiated) have been arbitrarily assigned a value of 100% and values for the irradiated samples have been expressed as a per cent of the controls.

C^{14}-glucose present in the extract was almost the same for both the X-irradiated samples and controls at both the 4- and 24-hour period. A number of unidentified compounds near the origin in the two-dimensional chromatograms were grouped together and classified as "origin area compounds". The activity in these was greatly decreased in the irradiated samples incubated with C^{14}-glucose 4 hours after X irradiation, but 24 hours after X irradiation they returned to the same value as the controls. The amino acids behaved somewhat similarly. The greatest change appeared in lactic acid. The 4-hour irradiated samples showed a great increase in activity in lactic acid over the controls. Twenty-four hours after X irradiation the irradiated samples incubated with C^{14}-glucose contained much less activity in lactic acid than did the controls.

Four hours after X irradiation the liver slices incubated with C^{14}-fructose incorporated less C^{14} into glucose than did the controls but in 24 hours there was more C^{14} in the glucose from the irradiated samples. Four hours after X irradiation the origin area compounds contained about the same amount of activity in both the irradiated and the non-irradiated samples incubated with C^{14}-fructose. The irradiated 24-hour samples showed a large increase in activity in the origin area compounds over the 24-hour controls. The amino acids behaved very much in the same manner. The incorporation of C^{14} into lactic acid, at both 4 and 24 hours after X irradiation, was greater in the irradiated samples than in the controls.

Discussion

The present study indicates that irradiation induces changes in the metabolism of glucose and fructose. However, at this stage of our study no conclusive interpretation can be given because of the lack of detailed knowledge on the mechanism of action of irradiation on metabolic processes.

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ON THE ANTIGENICITY OF INSULIN: FLOCCULATION OF INSULIN-ANTIINSULIN

P. J. Moloney and M. A. Aprile

Abstract

Serum from a horse which had received a series of injections of crystalline ox insulin showed typical flocculation with a solution of crystalline ox and pig insulins and with crude insulin extracted from the pancreas of the rabbit, horse, monkey, whale, and human. Flocculation was due to insulin and its corresponding antibody, since both insulin and antiinsulin, measured by mouse convulsion test, could be recovered from floccules. The ratio of insulin antibody to insulin (equivalence zone) is in the order of 497 to 553 micrograms per unit of insulin (40 μg). This corresponds to a molecular ratio of antibody: insulin of 3:1.

Introduction

The injection of crystalline pig or ox insulin into animals or humans can induce the formation of antibodies or antibody-like substances which can be made manifest in a number of ways, as for example, by certain in vitro reactions (1, 2, 3, 4, 5, 6) or by anaphylactic shock (7, 8, 9) or by neutralization of the physiological action of insulin on mixture with serum from an injected animal (10, 11, 8). The work of Lapresle and Grabar (12) has cast doubt on the relation of insulin to certain, at least, of the antibodies which may follow its injection. These investigators have shown that crystalline insulin may contain an active antigen which bears no relation whatever to insulin. Hence certain in vitro reactions and anaphylactic shock which have been ascribed to insulin–antiinsulin could in fact be due to non-insulin antigen–antibody. Further it might be suggested that neutralization of insulin by serum from an insulin-treated animal is due not to insulin antibodies but to destruction of insulin by induced proteolytic enzyme (insulinase). It is quite improbable, however, that this explanation of neutralization is correct since insulin may be recovered from insulin–antiinsulin mixtures (8, 13).

It is the purpose of this paper to demonstrate from evidence based on flocculation that insulin is indeed an antigen capable of inducing neutralizing antibodies.

Since in many antigen–antibody systems combination is exhibited by precipitation or flocculation, the possibility of such precipitation was explored in the case of insulin–antiinsulin. In all preliminary observations which we made using antiinsulin derived from the guinea pig, rabbit, sheep, or horse, precipitation was not observed. Finally, however, in mixtures of insulin and certain specimens of serum from a horse with induced insulin resistance, typical flocculation occurred.

Materials and Methods

Buffered saline. — Phosphate buffer (0.1 *M* Na_2HPO_4 + approx. 20 ml *N* HCl, pH 7.3–7.5) mixed with an equal volume of 0.85% (w/v) NaCl.

[1]Manuscript received January 20, 1959.
Contribution from Connaught Medical Research Laboratories, Toronto, Ontario.

Acid water. — Approximately 0.5 ml 6 *N* HCl to 1 liter distilled water (pH 2.5).

Insulin assay. — Estimates of insulin activity were obtained by mouse-convulsion assay (14).

Potency tests on sera. — Neutralizing activity was estimated by the prevention of hypoglycemic convulsions in mice (15).

Total nitrogen. — Micro-Kjeldahl method.

Vacuum distillations. — Flash-evaporator (Labline Inc., Chicago, Ill.).

Flocculating serum. — A summary of the course of immunization of the horse which yielded flocculating serum is given in Table I.

TABLE I

Response of horse No. 2589 to injections of insulin

Time, days	Units ox insulin injected	Potency of serum (units of insulin neutralized/ml of serum)
0	6250	
28	6250	
33		0.4
47		0.2
49	6250	
61		0.8 Lot A
70	6250	0.8 Lot B
85		0.8
92	6250	0.8
105		0.8 Lot C
112	6250	0.8
134		0.8 Lot D

NOTE: Insulin in Freund's adjuvant (8) was injected subcutaneously. Where blood was drawn and insulin injected on the same day (days 70, 92, 112) the taking of blood preceded the injection of insulin. Lots A, B, C, and D consisted of relatively large amounts of serum, 1 liter or more. Flocculation occurred in mixtures of insulin and serum of lots C and D, but not with serum of lots A and B.

Experimental

Lots C and D (Table I) were the sera with which flocculation was obtained. Table II shows the range over which flocculation occurred.

Data on the limiting concentrations of insulin and antiinsulin at which flocculation occurred are given in Table III.

TABLE II

Flocculation of insulin and antiinsulin

Units ox insulin in 0.5 ml	Antiserum "C" 1/3 dilution with buffered saline, ml	Flocculation
16	0.3	−
8	0.3	+
4	0.3	+
2	0.3	++*
1	0.3	+
0.5	0.3	−

NOTE: The mixtures were incubated in a water bath at 45° C. Flocculation occurred within 1 hour.
*This mixture was the first to show flocculation.

TABLE III

Limiting concentrations of insulin and antiinsulin for flocculation

Units ox insulin in 0.5 ml	Ml antiserum "C" in 0.3 ml				
	0.006	0.0125	0.025	0.05	0.1
4.0	−	−	++	++	++
2.0	−	−	++	++	++
1.0	−	+	++	++	++
0.5	−	+	++	++	++
0.25		+	+	+	+
0.125		tr	+	+	
0.0625		tr	−	−	

NOTE: Readings were made after incubation for 18 hours in a water bath at 45° C. + and ++ = flocculation, tr = very small amount of flocculation.

The equivalence zone was determined by testing supernatants after flocculation, for excess insulin and antiinsulin. Details of such determinations are given in Table IV.

TABLE IV

Equivalence zone of insulin–antiinsulin

Units ox insulin in 0.5 ml	Antiserum "C", ml	Flocculation	Flocculation in supernatants	
			Plus 0.025 ml antiserum "C"	Plus 0.25 units insulin
0.46	0.2	+	−	+
0.58	0.2	+	−	+
0.71	0.2	+	−	+
0.89	0.2	+	−	tr
1.0	0.2	+*	−	−
1.2	0.2	+*	+	−
1.4	0.2	+	+	−
1.7	0.2	+	+	−
2.1	0.2	+	+	−

NOTE: Two sets of mixtures consisting of constant amounts of serum "C" and of amounts of ox insulin which differed by increments of approx. 20% were prepared and left until flocculation had occurred (2 hours at 45° C). The mixtures were then centrifuged and each pair of supernatants was tested for excess insulin and antiinsulin by adding suitable small amounts of antiinsulin and insulin respectively, re-incubating, and noting the presence or absence of flocculation. Results in Table III served as a guide in choosing the suitable amounts of insulin and antiinsulin which were added.

For serum "C" the equivalence zone lies in the range 0.89–1.2 units insulin to 0.2 ml serum.

*These mixtures flocculated first.

That the flocculation was due to insulin and antiinsulin was shown by the fact that insulin and antiinsulin could be recovered in good yield from floccules.

Recovery of Antiinsulin from Floccules

Antiinsulin was recovered from insulin–antiinsulin floccules by a procedure which was first used by Pope and Healy (16) and later modified by Yeu and Pavageau (17) for the recovery of diphtheria antitoxin from toxin–antitoxin floccules.

To 4.0 ml of horse antiinsulin serum "C" was added 20 units of ox insulin (equivalence mixture, Table IV) in 10 ml buffered saline, pH 7.3. The mixture was left to flocculate at 45° C for 30 minutes, and then overnight at room temperature. The floccules were centrifuged, washed twice with 1-ml amounts

of cold saline, suspended in 3.0 ml saline, and the pH was adjusted to 3.2 with citric acid (5%). One milliliter 4% pepsin (pH 3.3) was added and the mixture maintained at 30–35° C for 1 hour. The pH was then adjusted to 5.2 (10% sodium acetate) and the mixture heated at 50° C for 15 minutes. The mixture was centrifuged at 5° C, the supernatant poured off, the pH adjusted to 7.5, and the volume made up to 8.0 ml. The antiinsulin titer of the solution by mouse convulsion test, was 0.2 units /ml. That is, total recovered antibody was $8 \times 0.2 = 1.6$ units. Antiinsulin serum "C" had a neutralizing titer of 0.8 units/ml or the 4 ml of serum used in the flocculating mixture had a total neutralizing power of $4.0 \times 0.8 = 3.2$ units. Thus the yield of antibody from the floccules was in the order of 50%.

Recovery of Insulin from Floccules and from Supernatants after Flocculation

Insulin, 2.4 units in 0.5 ml buffered saline, pH 7.5 was mixed with 0.4 ml horse antiinsulin serum "C" and left to flocculate at 45° C for 30 minutes. The floccules were centrifuged in the cold (5° C), washed once with 0.5 ml cold saline, and dissolved in 1 ml cold acid water (pH 2.5). Chilled absolute ethanol (3 ml) was added and the mixture left overnight in the cold (5° C). Water was added in an amount sufficient to reduce the alcohol concentration to 40%, the pH adjusted to pH 5.5 (10% sodium acetate), and the alcohol removed by vacuum distillation. The aqueous residue was assayed for insulin activity by the mouse convulsion method, yielding $> 25 < 50\%$ of total insulin originally in the flocculation mixture.

Insulin was extracted with acid ethanol (pH 2.5) from supernatants after flocculation. In detail, the supernatant (7 ml) from the flocculation of 10 units of insulin was combined with the first saline washing of the floccules (1 ml) and the pH adjusted to 2.5 with HCl. Ethanol was added to a concentration of 70% (v/v) and the mixture left at room temperature for 1 hour with occasional shaking. It was then centrifuged, the residue washed once with 1.5 ml of acid water, and the alcohol in the combined supernatant and washing was removed by vacuum distillation. (Final volume 6 ml.) Mouse assay of the aqueous residue showed that $> 15 < 30\%$ of the originally added insulin was in the supernatant after flocculation. That is, complete precipitation of insulin at the equivalence point was not observed.

Nitrogen in Floccules at the Equivalence Point

To six tubes containing 10 units insulin (2.5 ml 4.0 units/ml, pH 7.3) was added 2.5 ml saline and 2.0 ml horse antiinsulin serum "C". The mixtures were allowed to flocculate at 45° C for 30 minutes, then kept in the refrigerator for 3 days.

The floccules were washed three times with 1-ml amounts of cold saline, dissolved in 0.5 ml acid water (pH 2.5), and washed into micro-Kjeldahl flasks with acid water (final volume = 2.0 ml). Nitrogen was determined by the micro-Kjeldahl method.

The titer of 0.01 *N* HCl for six determinations was 3.35 ± 0.07 ml, giving total N = 470 μg.

Diffusion of Insulin and Antiinsulin in Agar Gel

Diffusion was carried out according to the technique of Ouchterlony (18). Small metal chimneys were placed approximately 20 mm apart in Petri dishes containing a layer of 1% agar (5–6 mm thick). To the center chimney was added 0.2 ml of a 1:1 mixture of saline and horse antiinsulin serum "C"; to the outer chimney was added 0.2 ml (equivalence amount) of insulin (2.5 units/ml, pH 7.5). The Petri dishes were left for 7–10 days at room temperature in a closed vessel containing physiological saline (0.85% NaCl), after which time a single curved white precipitin line had formed near the antiinsulin reservoir and slightly curved towards it (Fig. 1).

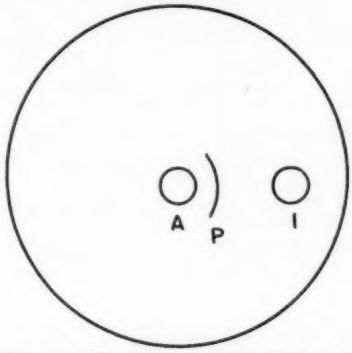

FIG. 1. Diffusion of insulin and antiinsulin in agar gel.
A: 0.2 ml antiinsulin serum, diluted 1:1 with saline.
I: 0.2 ml insulin (2.5 units/ml, pH 7.5).
P: precipitin line.

Discussion

Blood serum of a horse taken 105 days after the first of a series of injections of insulin was capable of neutralizing the physiological action of insulin and also showed, on mixture with insulin, the phenomenon of flocculation. Earlier specimens of serum from the same horse showed neutralization only. That flocculation was due to insulin and its corresponding antibody was shown by the fact that both insulin and antiinsulin, each assayed by mouse method, not by flocculation, could be recovered from floccules.

It was found that appreciable amounts of insulin (15–30% of the total) could be recovered from supernatants of equivalence zone mixtures of insulin and antiinsulin after flocculation had taken place. Certain observations suggest that incomplete precipitation of insulin–antiinsulin depended, in part at least, on the presence of non-precipitating antibody in the flocculating serum. It was found that flocculating serum could be fractionated into two portions by precipitation with ammonium sulphate (10 ml serum + 30 ml water + 7 g $(NH_4)_2SO_4$). The two portions, supernatant and precipitate, when tested after removal of ammonium sulphate, were each found to contain approximately 50% of the original insulin-neutralizing activity; the one which was precipitated by ammonium sulphate flocculated on mixture with insulin, the other did not.

There were differences in amounts of insulin recovered from floccules and supernatants after flocculation with whole serum and with the ammonium-sulphate-precipitated fraction. More insulin was precipitated by the ammonium-sulphate-precipitated fraction than by unfractionated serum. This is indicated by the amounts of insulin extracted from floccules and from supernatants and by the amount of floccules (in terms of nitrogen) precipitated (Table V). These results are in agreement with the assumption that incom-

TABLE V

Floccule analysis

	Insulin recovery, %		Floccules per 10 units insulin	
	From supernatant	From floccules	Total nitrogen, μg	Total protein (calc.), μg
Flocculation with whole serum	>15<30	>25<50	470	3030
Flocculation with ammonium-sulphate-pptd. fraction	>9.0<13.0	>50<69	740	4770

NOTE: The assay of insulin extracted from the supernatant and from floccules of the ammonium-sulphate-precipitated serum was more accurate (larger numbers of mice used in the tests) than the corresponding assays in the case of the whole serum.

For the ammonium-sulphate-precipitated serum, the flocculating mixture contained 108 units insulin; 11.5 units (confidence limits: 9.5–14.0 units) were extracted from the supernatant and 63.6 units (confidence limits: 53–74 units) were extracted from the floccules. Percentage recoveries are shown in the table.

plete precipitation of insulin is related to the presence in the flocculating serum of non-flocculating insulin antibody.

The amount of flocculating antibody which corresponds to a unit of insulin may be calculated from the data given in Table V provided that the floccules consist essentially of insulin and its corresponding antibody and that the amount of insulin in the floccules is known.

Results of agar diffusion (Fig. 1) support the assumption that the floccules consist essentially of one antigen–antibody system. One line of precipitation only was observed and the position and curvature of the line was consistent with the diffusion of smaller molecular weight insulin (mol. wt. 36,000) toward larger molecular weight antibody (horse globulin, mol. wt. 160,000).

Before calculating the amount of insulin in floccules the question of loss must be considered. Loss of antigen (and antibody) in floccules might possibly occur as a result of the washing which is carried out preparatory to nitrogen determinations. This possibility was checked for insulin–antiinsulin floccules by washing with cold saline, two lots of floccules three times and two other lots five times. The amounts of the washed floccules in terms of micrograms of nitrogen were: 470, 467 and 464, 460 respectively. That is, washing brought about no appreciable loss of floccules.

Results in Table V show that the yields of insulin from floccules plus supernatants were less than 100%. In calculating the amount of insulin in the floccules it was assumed that extraction of insulin was equally good from floccules and from supernatants. On this assumption the amount of insulin in floccules from a total of 10 units would be:

$$\frac{\text{Extracted insulin from floccules}}{\text{Extracted insulin from floccules plus supernatant}} \times 10.$$

Calculations gave the following values for units of insulin in floccules per 10 units in flocculating mixture:

Insulin in insulin – whole serum floccules:

$>4.5 <7.7$ units

or $>180<308$ μg (assuming 40 μg/unit)

Insulin in insulin – ammonium-sulphate-precipitated fraction floccules:

$>8.0 <8.9$ units

or $>320 <356$ μg

From the above the amount of antibody in floccules may be calculated. Results are shown in Table VI.

TABLE VI

Insulin and antibody protein in floccules

	Insulin		Insulin + antibody, μg	Antibody, μg
	Units	μg		
Insulin – whole serum floccules	$>4.5<7.7$	$>180<308$	3030	$>2722<2850$ Av. 2786
Insulin – ammonium-sulphate-pptd. fraction floccules	$>8.0<8.9$	$>320<356$	4770	$>4414<4450$ Av. 4432

NOTE: Units recorded are total units of insulin in floccules calculated from extracted units given in Table V.

From Table VI the limiting values in micrograms of antibody protein per unit of insulin are: $>362<619$ and $>497<553$. These latter values are used in the calculations.

In the equivalence zone of flocculation, whole serum neutralized 5 units of insulin per ml. Hence the antibody content of the serum was $>5\times497$ and $<5\times553$ μg per ml.

Boyd and Hooker (19) have derived the following formula for the relation between ratio of antibody to antigen at the equivalence point and the molecular weight of the antigen:

$$\log \frac{\text{antibody}}{\text{antigen}} = 3.49 - 0.529 \log \text{mol. wt. antigen.}$$

Substitution in this formula of values for the ratio of antiinsulin: insulin = 497/40 and 553/40 gives the molecular weight of insulin as 28,000–34,000, which is in the right order of magnitude for insulin in aqueous solution, pH 7.8.

If we assume molecular weights of antibody (horse globulin) and insulin to be 160,000 and 36,000 respectively, the molecular ratio of antiinsulin: insulin in equivalence floccules is 3:1.

The horse antiinsulin flocculating serum gave typical flocculation not only with ox insulin which had been used to induce the antiinsulin but also with

crystalline and crude pig insulins and with insulins extracted from the pancreas of the rabbit, horse, monkey, whale, and human. The horse antiinsulin neutralized all these extracted insulins, as shown by mouse test.

The ratio of insulin to antiinsulin, as measured by flocculation in the equivalence zone, differs rather markedly from the ratio of insulin to antiinsulin as measured by the mouse convulsion method, viz. 5 units insulin to 1 ml serum ("C") and 0.8 units insulin to 1 ml serum, respectively. That is, more antiinsulin is required to neutralize insulin by animal test than corresponds to an equivalent mixture by flocculation.

An analogous result has been observed with scarlet fever toxin–antitoxin (20, 21). The explanation of the phenomenon with insulin–antiinsulin is probably that which has been advanced for scarlet fever toxin–antitoxin, namely that the dissociation constant for soluble insulin–antiinsulin complex is large and hence excess antitoxin is necessary to effect neutralization.

Acknowledgments

The authors gratefully acknowledge the assistance of Miss Anne Tirpak. We also acknowledge with thanks the financial assistance given by the J. P. Bickell Foundation.

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NOTES

ATTEMPTED REVERSAL OF THE DIETHYLSTILBESTROL INHIBITION OF SUBSTRATE OXIDATION IN AEROBACTER AEROGENES*

NORMAN N. DURHAM AND MARGIE D. PERRY

It has been reported that diethylstilbestrol (4,4'-dihydroxy-α,β-diethylstilbene) inhibited the succinoxidase system of animal tissues and this inhibition could be reversed if brilliant cresyl blue (1) or methylene blue (2) was incorporated in the system. Reilly (3) reported that inhibition of malate oxidation by diethylstilbestrol was reversed when cytochrome *c* and diphosphopyridine nucleotide were added. It was suggested that cytochrome oxidase and succinic dehydrogenase are sensitive to the synthetic estrogen. Earlier, Hochster and Quastel (4) had proposed that diethylstilbestrol can exist in a quinone form and may then influence various dehydrogenase systems. Further studies have shown that diethylstilbestrol suppresses the rate of oxygen uptake by cell suspensions of *Aerobacter aerogenes* with glucose, maltose, fructose, succinate, and pyruvate as substrates (5).

To elucidate the effect of diethylstilbestrol on *A. aerogenes*, reversal studies were carried out with washed cell suspensions and cell-free extracts. The cell suspensions were prepared by growing the organism on nutrient agar at 37° C for 18–20 hours, washing the cells twice, and resuspending them in *M*/120 phosphate buffer of pH 7.0. Crude extracts were prepared by grinding the cells with glass beads in a Virtis homogenizer and centrifuging at 5000 × *g* for 30 minutes. All experiments were performed at 37° C and the test compounds were studied over a wide range of concentrations.

Manometric studies with whole cells in the presence of diethylstilbestrol indicated that neither brilliant cresyl blue nor methylene blue reversed the depressed rate of oxygen uptake with maltose, fructose, glucose, succinate, oxalacetate, or pyruvate as substrates. Figure 1 shows results obtained with succinate. Results from appropriate controls showed that the rate of oxygen uptake was not influenced by the addition of brilliant cresyl blue or methylene blue. Data also indicated that diethylstilbestrol did not influence the endogenous respiration of the cell suspension. Diphosphopyridine nucleotide (0.1 mg), adenosine triphosphate (0.2 mg), and cytochrome *c* (0.1–0.2 mg) were studied in the presence and absence of the dye compounds. Similar experiments were performed in which ions of manganese, calcium, and magnesium were added to the system. These additives caused little, if any, reversal in the suppression of oxygen uptake. Additional studies indicated that yeast extract, casamino acids, or vitamin-free casein hydrolyzate were also ineffective in reversing the effects of diethylstilbestrol.

*This work was supported in part by Oklahoma Agricultural Experiment Station, Project No. 976, and in part by a grant-in-aid from Chas. Pfizer and Co., Inc.

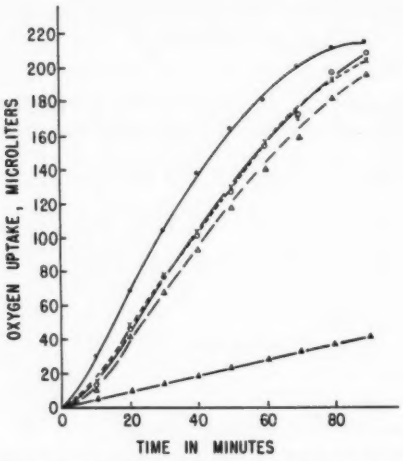

FIG. 1. The oxidation of succinate in the presence and absence of diethylstilbestrol and added electron acceptors.
▲, endogenous; ●, succinate (2.5×10^{-5} *M*); ×, succinate and diethylstilbestrol (1.3×10^{-4} *M*); ○, succinate, diethylstilbestrol, and brilliant cresyl blue (1:132,000); △, succinate, diethylstilbestrol, and methylene blue (1:220,000).

The enzymatic activity of cell-free extracts was studied by measuring the rate of dye reduction in Thunberg tubes. Results obtained from experiments in which glucose, succinate, and pyruvate were employed as substrates showed that diethylstilbestrol suppressed the rate at which both methylene blue and brilliant cresyl blue were reduced. Attempts to reverse the inhibition with 2,3,5-triphenyl tetrazolium, cytochrome *c*, diphosphopyridine nucleotide, and/or riboflavin-5-phosphate were unsuccessful.

These data show that the rate of oxygen uptake with several substrates by whole cells and cell-free extracts of *A. aerogenes* is suppressed by diethylstilbestrol and that this suppression is not reversed by the addition of various organic metabolites, electron carriers, or metallic ions which function as enzymic cofactors. It is suggested that diethylstilbestrol may not function by competing directly with hydrogen carriers in this organism but may function by a different mechanism such as diverting the electrons from molecular oxygen and the artificial acceptors or serving as an electron trap.

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CORRECTIONS

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Page 219. In Table I, opposite "Potassium, mM./kg.", the values 15 ± 2 (4) 20 ± 5 (5) should read 20 ± 5 (5) 15 ± 2 (4).

Pages 377, 378. On p. 377, line 25, and on p. 378, Table IV, Galactose-U-C^{14} should read Galactose-1-C^{14}.

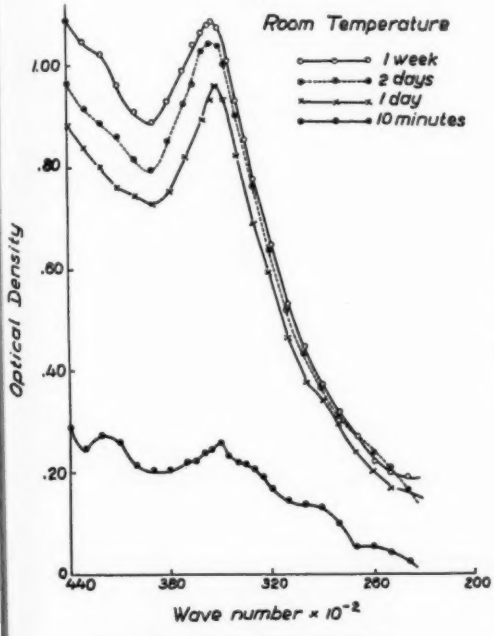
Page 959. In the Abstract, line 14, the sentence "The rate of repolarization was decreased . . ." should read "The rate of repolarization was increased . . .".

Page 1181. The first column of Table I should appear as follows:

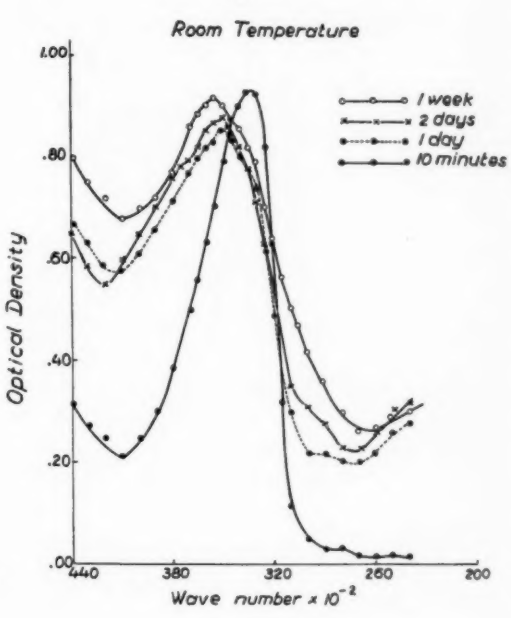
(1) Albumin* Conjugate
(2) Albumin Conjugate
(3) Albumin Conjugate
(4) Albumin Conjugate
(5) Albumin Conjugate

Pages 1227, 1228. The lower figures on these two pages should be transposed; the figure that appears on p. 1227 is 4*b* and the figure on p. 1228 is 3*b*, as indicated below.

3*b*



4*b*



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Page 28. In column 3 of Table I, opposite Experiments 1, 3, and 5, the prefix "DL-" should be omitted before the abbreviation "*p*-HPPA".

Page 289. The legend on the ordinate of Fig. 2 should read

Percent C^{14} infused per min
found per mg carbon $\times 10^{-2}$.

NOTICE

The Endocrinology Study Section, National Institutes of Health, announces that it is prepared to distribute, free of charge to qualified investigators, limited amounts of purified sheep pituitary follicle-stimulating hormone. The preparation has been packaged in two forms: (1) vials containing 5 mg for use only as a Standard in assay procedures; (2) vials containing 25 mg, for experimental use. The preparation is a sterile, lyophilized powder, free or nearly free of other hormonal activities. Detailed specifications will be distributed with each sample.

Application for supplies, either of the Standard or of the experimental lots, should be made by letter to Dr. R. T. Hill, Executive Secretary, Endocrinology Study Section, National Institutes of Health, Bethesda 14, Maryland. The letter should state briefly an outline of the intended work and an estimate of the amount needed. Applications will be screened by a subcommittee and allotments will be made according to the estimates of merit of the intended work.

The preparation and distribution of FSH is supported by funds granted by several Institutes of the National Institutes of Health. The program is supervised by a subcommittee consisting of Drs. Robert W. Bates, Roy W. Hertz, R. T. Hill, Warren O. Nelson, and Alfred E. Wilhelmi, Chairman. The hormone preparation was made at Emory University under the immediate direction of Dr. Stanley Ellis.

U. S. Department of
Health, Education, and Welfare,
Public Health Service,
National Institutes of Health,
Bethesda 14, Maryland.

February 2, 1959.

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